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OF
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S. E. JACOBS AND D. J. JAYNE-WILLIAMS

WITH THE ASSISTANCE OF

E. BILLING
L. F. L. CLEGG
E. L. CROSSLEY
B. P. EDDY
N. D. HARRIS
R. M. KEDDIE

L. J. MEANWELL
J. MEIKLEJOHN
G. SYKES
C. C. THIEL
S. B. THOMAS
H. R. WHITE

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HUSAIN, I., HALL, A. N. & WALKER, T. K. (1960). *J. appl. Bact.* **23** (1), 1–6.

A MUCILAGE-FORMING *LACTOBACILLUS* SPECIES ISOLATED FROM CIDER.

PART II. POLYSACCHARIDE FORMATION

BY I. HUSAIN, A. N. HALL AND T. K. WALKER

Manchester College of Science and Technology, Manchester

SUMMARY: Cultures grown at 25° under an atmosphere of CO_2 in a medium containing maltose, inorganic salts and ethanolic extracts of peptone and yeast autolysate produced a polysaccharide, which was isolated and purified. Examination by a variety of chemical and biological methods indicated that it was a dextran of low molecular weight.

IN A RECENT communication (Millis, Husain, Hall & Walker, 1958) an account was given of the principal morphological, physiological and biochemical characteristics of a heterofermentative *Lactobacillus* species isolated from cider. It showed similarities to *L. pastorianus* and to *L. brevis*, and one of its most interesting features was an ability to produce high levels of viscosity in maltose-containing media; growth and the formation of viscous products were greatest when cultures were incubated under an atmosphere of carbon dioxide.

The present report is specifically concerned with the isolation of a polysaccharide from cultures grown in a medium containing maltose. A considerable amount of preliminary work (Husain, 1954) was involved in a search for a polysaccharide-free medium capable of supporting strong growth and the development of high levels of viscosity. This step was essential in order that the bacterial polysaccharide could be isolated readily by precipitation with ethanol.

MEDIA AND CULTURAL CONDITIONS

Preliminary work. The failure of the organism to grow in synthetic media containing amino acids, vitamins and purines, as described by Dunn *et al.* (1947) and by MacMahan & Snell (1944), pointed to a complex growth requirement. The nitrogenous constituents used in the preparation of complex media were obtained by extracting peptone with 96% (v/v) ethanol or by adding ethanol to yeast autolysate to give a final ethanol concentration of 90% (v/v), the ethanol insoluble fractions being rejected in each case.

Polysaccharide-free media based on ethanolic extracts of green malt extract and on various combinations of the ethanolic extracts of casein hydrolysate, of yeast autolysate and of peptone were tested and the conclusion was reached that a medium containing extracts of peptone and of yeast autolysate was superior to the other media in supporting growth of the organism and the development of the viscous condition.

Peptone extract. This was prepared as described by Hopton & Hall (1959).

Yeast autolysate extract. This was prepared essentially according to the instructions of Hopton & Hall (1959); the extract, however, was not subjected to acid hydrolysis.

Growth medium. The medium used in the preparation of bacterial polysaccharide contained: KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 1 g; $FeSO_4.7H_2O$, 0·01 g; maltose, 20 g; peptone extract equivalent to 1 g of nitrogen and yeast autolysate extract equivalent to 1 g of nitrogen in distilled water to 1 litre. The pH value was adjusted to 5·1–5·2.

Cultural conditions. Pyrex aspirators (5 litres capacity), fitted with rubber stoppers (and taps) at the base and cotton wool plugs at the top, were each charged with 3 litres of growth medium and sterilized in an atmosphere of free steam for 20 min on each of 3 successive days. Each aspirator was then inoculated with 8 ml of a 4 day culture of the organism in the growth medium. The cotton wool plugs in the aspirators were replaced by sterile rubber stoppers fitted with inlet and outlet taps and the aspirators were flushed with a stream of sterile CO_2. The cultures were then incubated at 25° and samples were withdrawn at intervals to follow the increase in viscosity to a maximum. This value was usually attained after 13–14 days, when the maltose content of the medium had fallen to about 0·2% (w/v). After sampling, the atmosphere in the aspirators was replenished with fresh supplies of sterile CO_2.

ANALYTICAL METHODS

Reagents. All reagents used were of Analar or equivalent quality.

Ash. Weighed amounts of polysaccharide were charred in a platinum crucible, heated gradually to 800° and maintained at that temperature for 2 hr. The weight of ash was then determined.

Nitrogen. Measured quantities of material were digested with H_2SO_4 in the presence of $KHSO_4$ and selenium catalyst. After digestion for 2 hr the NH_3 was liberated and distilled in a Markham apparatus (Markham, 1942), being collected in the boric acid-indicator mixture described by Kabat & Mayer (1948).

Phosphate. The method of Allen (1940) was employed.

Glucose and maltose. The semi-micro method of Somogyi (1945) was used.

Hydrolysis of polysaccharide. A 200 mg quantity of polysaccharide was dissolved in 15 ml of N H_2SO_4 and heated in a sealed tube for 6 hr at 100°. On cooling, the solution was neutralized with excess $BaCO_3$ and the inorganic material centrifuged down. The supernatant and washings were then accurately made up to a known volume.

Paper chromatography of sugars. Samples were chromatographed on Whatman no. 1 filter paper in an ethyl acetate-acetic acid-water solvent system (Jermyn & Isherwood, 1949). Reducing sugars were detected by means of the alkaline silver nitrate reagent of Trevelyan, Procter & Harrison (1950).

Specific rotation. Readings were made on a 0·5% (w/v) solution of the polysaccharide in 0·5N NaOH; a few drops of formamide were added to reduce the opalescence of the solution.

Molecular weight. Purified polysaccharide (300 mg) was dissolved with trituration in 4 ml of 6N NaOH, the resulting solution then being diluted with water and neutralized with N HCl. The polysaccharide solution was next dialyzed against running water to remove traces of low molecular weight carbohydrates and of inorganic salts. The polysaccharide was then isolated by evaporation of the solution to dryness *in vacuo*. The degree of polymerisation and hence the molecular weight of the polysaccharide was then estimated by the reducing end-group method of Meyer *et al.* (1949).

Blue value. The determination was made in accordance with the recommendations of Bourne *et al.* (1948).

Metaperiodate oxidation. Finely powdered polysaccharide (180 mg) was dispersed in freshly boiled distilled water (200 ml) and the suspension distributed equally between 20 Erlenmeyer flasks (25 ml capacity). To the contents of each flask 0·5 ml of 0·3 M sodium metaperiodate solution was then added, the flasks being stoppered, stored at 25° in the dark and shaken periodically. At intervals of 24 hr the contents of 2 flasks were separately treated with 0·5 ml of ethylene glycol and allowed to stand for 60 min. Formic acid was then estimated by titration against 0·01N NaOH to phenolphthalein in an atmosphere of carbon dioxide-free nitrogen. Flasks containing polysaccharide solution, without the addition of periodate, served as controls.

Reaction with α-amylase. Pure polysaccharide (50 mg) was dissolved, by heating to 60–70°, in 30 ml of 0·15M NaCl. The polysaccharide solution was then divided into 10 equal portions distributed between Erlenmeyer flasks of 10 ml capacity, and 1 ml of a 1:8 dilution of saliva at pH 6·0 was added to each portion. Finally the flasks were incubated at 37° for 20 hr and the reducing sugar liberated was estimated and expressed as maltose.

RESULTS

Isolation of the crude polysaccharide

The viscous culture fluid was clarified by Seitz filtration and the polysaccharide precipitated by the addition of 3 volumes of ethanol (96% v/v). The supernatant fluid was syphoned off as far as possible and the fibrous product collected at the centrifuge. The polysaccharide was then washed by suspension in a 3:1 (v/v) mixture of ethanol and water, centrifuged down, washed twice more and finally dried *in vacuo* over $CaCl_2$.

From a series of preparations made over a period of several months from a total of about 65 litres of culture medium 3·5 g of product were isolated. On analysis it gave: ash, 3·7%; nitrogen, 0·87%; and reducing sugar produced on hydrolysis, 47% (expressed in terms of the theoretical yield of glucose obtained on hydrolysis of a polyglucose). Chromatographic examination of the hydrolysate showed glucose to be the only reducing sugar present.

Purification of the polysaccharide

Whereas freshly precipitated crude polysaccharide was freely soluble in water, this property was lost after storage for several days over $CaCl_2$. Purification of the dried product was, however, readily effected by dissolution in N HCl and subsequent precipitation as a fine, white, amorphous powder by addition of the solution in a dropwise manner, with continuous stirring, to absolute ethanol (4 vol). The precipitate was collected at the centrifuge and washed several times with absolute ethanol.

The regenerated polysaccharide was freely soluble in water and accordingly was dissolved in that solvent, when it yielded a viscous solution from which it was recovered by adding it dropwise to 3 volumes of absolute ethanol. The product was further purified, on two other occasions, by precipitation from aqueous solution with 3 volumes of ethanol and finally with 2 volumes of ethanol. The white product (1·3 g) was stored over $CaCl_2$ *in vacuo*; a procedure which, according to Jeanes, Wilham & Miers (1948), is sufficient to eliminate the last traces of ethanol.

Analysis and characterization of the polysaccharide

Rotation. The value of $[\alpha]_D^{20}$ was 220°.

Elementary analysis. Found: C, 43·9%; H, 6·17% (Drs. F. & E. Pascher, Vienna). $(C_6H_{10}O_5)_n$ requires C, 44·4%; H, 6·17%. The ash content was 0·17% and nitrogen and phosphate were not detected.

Glucose formed on hydrolysis. The yield of glucose obtained was 99·0% of theory.

Molecular weight. A value of 43 was obtained for the degree of polymerisation (mean of 3 determinations), which corresponds to a molecular weight of about 7,000.

Metaperiodate oxidation. The formic acid liberated was 0·95 mole/mole of anhydroglucose, which indicates (within experimental error) the existence of a ratio of 1;6– to 1;4– linkages of about 20:1.

Infrared spectrum. This showed a peak at 838 cm^{-1}, which is indicative of the presence of linkages of the α type, and peaks at 914 cm^{-1} and 768 cm^{-1}, which show the presence of 1;6– linkages.

Blue value. The polysaccharide did not give a colour reaction with iodine.

Reaction with α-amylase. The conversion to reducing sugar was less than 0·6%.

Serological reactions. The polysaccharide reacted strongly with antiserum to pneumococcus type II in a dilution of 10^{-2} but no detectable precipitation occurred with solutions more dilute than 10^{-4}. On the other hand a moderately strong reaction occurred in dilutions up to 10^{-6} between the polysaccharide and antiserum to *Leuconostoc mesenteroides*. It thus appears that the polysaccharide is serologically related to the dextran of *Leuc. mesenteroides*.

Detection of glucose in cultures

Samples of culture fluid withdrawn after 3, 5 and 7 days were examined by paper chromatography. In addition to maltose, considerable amounts of glucose were detected at 3 and 5 days, and smaller amounts at 7 days. A control prepared from uninoculated medium containing lactic and acetic acids in the proportions in which they were likely to be formed in cultures (Millis *et al.* 1958) was incubated for 7 days and then examined. It did not show more than a trace of glucose.

DISCUSSION

The examination of the polysaccharide by physical methods provided considerable evidence that the compound is a dextran, the high positive rotation and the infrared spectrum being in accordance with this view. The results of the biological tests, including the cross reaction with *Leuconostoc* antiserum and the failure to react with α-amylase, are also consistent with the view that the polysaccharide is a dextran.

In the absence of information relating to the mechanism of polysaccharide formation, alternative views are perhaps worthy of consideration: first, that polysaccharide synthesis is dependent on a phosphorolytic cleavage of maltose to glucose and a glucose-l-phosphate which is the polysaccharide precursor, and second, that α-glucosyl radicals are transferred from maltose to glucose, or to other acceptor molecules. Indeed, the detection of a small proportion of 1;4– linkages in the polysaccharide molecule is consistent with the possibility that one molecule of maltose serves as the glucosyl acceptor in the synthesis of one molecule of polysaccharide. It is perhaps relevant to note, in this connection, that Koepsell *et al.* (1953) have shown that maltose can serve as a glucosyl acceptor in the dextran sucrase-sucrose reaction.

In recent years the synthesis of oligosaccharides from maltose, by the process of trans-α-glucosylation, has been demonstrated by the use of enzyme preparations isolated from moulds, bacteria, marine algae and animal tissue. The position has recently been summarized by Duncan & Manners (1958). These authors pointed out that whereas the mould enzymes readily transfer α-glucosyl radicals from maltose to the primary alcohol group of glucosaccharides to create 1;6–α–linkages, the enzymes of *E. coli* and of rat liver lead to the synthesis of 1;4–α–glucosidic linkages. According to Duncan & Manners, enzyme extracts from the marine alga *Cladophora rupestris* transfer α-glucosyl residues from maltose to hydroxyl groups at $C_{(4)}$ or $C_{(6)}$ of the non-reducing end-group in maltose or maltotriose. The results described in the present paper suggest the possibility that the mucilage-forming *Lactobacillus* species isolated from cider might well be a useful source of transglucosylases possessing the ability to synthesize the 1;6–α–glucosidic linkage.

Through the kind offices of Professor H. B. Maitland the serological tests were carried out in the Department of Bacteriology, University of Manchester, by Dr. Eve Billing, to whom we express our appreciation. Thanks are also due to Dr. S. A. Barker of the University of Birmingham for the infrared spectroscopy.

The present address of I. Husain is: The University, Aligarh, U.P., India.

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A TRIPLE SUGAR-UREA-IRON MEDIUM FOR THE CONFIRMATION OF *SALMONELLA* AND THE EXCLUSION OF *PROTEUS* STRAINS

By D. J. STEWART

Birds Eye Foods Ltd., South Denes, Great Yarmouth, Norfolk

SUMMARY: A modification of the dulcitol-lactose-iron medium of Taylor & Silliker (1958) is proposed. It contains sucrose as well as lactose to aid in detecting slow lactose fermenters and gives simultaneously tests for urea hydrolysis and H_2S production. The latter reaction is kept apart from the carbohydrate fermentations and the urea hydrolysis. The surface growth can be used in a test for the deamination of phenylalanine to phenylpyruvic acid.

The isolation and identification of *Salmonella* is one of the more important problems facing food bacteriologists. Isolation by enrichment and plating on selective media, the method in general use, leads to the point where serological and/or biochemical tests must be applied to any organism which in its colonial appearance resembles a *Salmonella*.

The biochemical tests usually entail the inoculation of six or seven different media, a procedure which is both time and material consuming. Many short cuts to the biochemical differentiation of members of the Enterobacteriaceae have been proposed, and various complex media, such as Russell's double sugar agar, Krumwiede's triple sugar agar, Kligler's iron agar, etc., are widely used because they permit two or three biochemical reactions to be determined in one culture. Most recently, Taylor & Silliker (1958) developed a complex medium in which an organism's ability to ferment dulcitol and lactose and to form H_2S were all demonstrable in one tube. In essence, this dulcitol-lactose-iron (DLI) agar is Kligler's iron agar modified by the substitution of dulcitol for glucose, and the medium is poured in two layers so as to confine the production of H_2S to the upper layer; by so doing the dulcitol reaction (in the lower layer) is not obscured. Dulcitol was preferred to glucose by these workers, because most *Salmonella* spp. ferment dulcitol, whereas *Proteus* spp. and most of the 'paracolon' group do not. In view of this 'negative' test for *Proteus*, it seemed that an improvement might be effected by the inclusion of a 'positive' test, such as that for the production of urease, the classical test for *Proteus*. This was accomplished, and the composition, preparation and use of the modified DLI medium are briefly described below.

Preparation and Inoculation of the Medium

Many modifications were tried, and the one described below proved the best. It is a triple sugar-urea-iron formulation, sucrose being included to detect those slow lactose fermenters which often ferment sucrose much more rapidly. The medium is used in two layers in tubes, the upper layer providing a sloped surface, and there is a two-layered butt. The upper, larger layer contains the carbohydrates and the iron salt and the lower layer the urea and the thiosulphate. The H_2S reaction is thus confined to the region of contact between the layers and does not obscure either the carbohydrate reactions or the urea hydrolysis. The growth on the surface of the upper layer may be washed off and used in a test of its ability to convert phenylalanine to phenylpyruvic acid.

Preparation. The lower layer, or urea base, is similar in composition to the medium of Stuart, Rustigian & Van Stratum (1945), with the addition of agar, sodium thiosulphate and urea. The following ingredients are dissolved, with heat, in 95 ml of distilled water and the solution sterilized at 15 lb/in^2 for 15 min: $Na_2HPO_4.12\ H_2O$, 0·9 g; KH_2PO_4, 0·9 g; Difco 'Bacto' yeast extract, 0·01 g; $Na_2S_2O_3.5H_2O$, 0·03 g; agar (Oxoid), 2·0 g; bromothymol blue, 0·01 g. This medium is then cooled to 50° and 5 ml of a 40% sterile urea solution added. After thorough mixing, 2 ml amounts are aseptically dispensed into sterile plugged 15 × 125 mm test tubes and allowed to set. These prepared tubes will keep for several months without deterioration, even when stored at room temperature.

To prepare the upper layer, the following ingredients are first dissolved, with heat, in 480 ml of distilled water and the pH adjusted to 7·4: Difco 'Bacto' phenol red agar base, 15·5 g; lactose, 5·0 g; sucrose, 5·0 g; agar (Oxoid), 2·5 g; $(NH_4)_2SO_4.FeSO_4.6H_2O$, 0·1 g. After sterilization at 15 lb/in^2 for 15 min the medium is cooled to 50° and 20 ml of a sterile 5% dulcitol solution added, to give a final dulcitol concentration of 0·2%.

To each tube of urea base is now added 6–7 ml of this triple sugar medium and the tube sloped to form a butt of about 1 in. in the upper layer. The complete medium should be used on the same day as the upper layer is added.

Inoculation. The very centre of a suspect *Salmonella* colony is picked off the selective plate with a straight wire and streaked and stabbed into a tube of the triple sugar-urea-iron medium, ensuring that the stab goes right to the bottom of the lower butt and that the slope is well streaked to obtain good growth. The cultures are then incubated for 18–24 hr at 37°, after which the following reactions may be read: urea hydrolysis, shown by the change of the lower butt from green to an intense blue; H_2S production, shown by the development of a narrow ($\frac{1}{4}$ in.) black ring at the junction of the lower and the upper layers; dulcitol fermentation, shown by the change of the upper butt from orange-red to yellow, with or without disruption of the agar by gas formation, and lactose/sucrose fermentation, shown by the change of the slope from orange-red to yellow.

RESULTS

Performance of the medium. Over fifty strains from groups in the Enterobacteriaceae were used to test the reliability of the triple sugar-urea-iron (TSUI) medium, including 10 strains of *Salmonella typhimurium*, 6 of *Salm. enteritidis*, 12 of other salmonellas, 10 of *Proteus* spp., 10 of 'paracolon' types, 4 of *Escherichia coli* I and 3 of *Aerobacter aerogenes*.

The biochemical reactions of these organisms on the TSUI medium were compared with those on Difco phenol red agar base plus 1% of lactose, 1% of sucrose or 0·2%

of dulcitol, Stuart's urea medium plus 2·0% of agar and Kligler's iron agar. In every case, the results obtained on the TSUI medium were the same as those on the simple single test media.

The phenylalanine test on the surface growth. The reliability of the urea test for differentiating *Proteus* has been questioned by Buttiaux, Moriamez & Papavassiliou (1956), who found that an organism's ability to deaminate phenylalanine to phenylpyruvic acid was a more definite and specific characteristic of *Proteus* strains. It was already known that this 'phenylalanine test' could be done with cells harvested from a slope culture (Ben Hamida & Le Minor, 1956), and in fact cells harvested from a well streaked Kligler's iron agar slope serve, thus extending the use of that medium (Stewart, 1959). Similarly, cells harvested from a TSUI slope may be used, as follows.

After reading the fermentation, urea and H_2S reactions, the growth on the slope is emulsified, with the aid of a wire loop, in 0·5 ml of a 0·2% solution of L-β-phenylalanine in 0·1 M, pH 7·2, Sørensen buffer and the resulting suspension poured into a ¼ oz 'bijou' bottle which is firmly stoppered and shaken on a 'Microid' flask shaker for 15 min. Five drops of an 8% aqueous solution of $FeCl_3$ are then added and the bottle reshaken for a further 20 sec. If phenylpyruvic acid has been formed an intense green colour develops. This reaction must be read at once as the colour soon fades.

General remarks. The TSUI medium would seem to allow at least four biochemical reactions to be determined in one tube, with no interference between them, thus permitting the identification of *Proteus* by a urea hydrolysis test. This can be checked by a phenylalanine test, using the surface growth. The medium is no more difficult to prepare than the DLI medium of Taylor & Silliker (1958).

The present address of the author is The Ministry of Agriculture for Northern Ireland, Bacteriology Division, Elmwood Avenue, Belfast, N.I.

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THE SANITATION OF FISH BOXES. II. THE EFFICIENCY OF VARIOUS SANITIZERS IN THE CLEANING OF COMMERCIAL WOODEN FISH BOXES

BY R. SPENCER

Humber Laboratory, Department of Scientific and Industrial Research, Hull

SUMMARY: An investigation of the effect of various sanitizers, including kinds containing hypo-chlorite, formaldehyde and quaternary ammonium compounds, alone and with detergents, and of a nonionic detergent alone, on the cleanability by various methods of commercial wooden fish boxes, showed that no major reductions in viable counts greater than 99·9% could be expected; this was due presumably to the high degree of contamination of the box and the porous nature of the wood. Of the materials investigated, hypochlorite solutions containing not less than 1,000 p/m of free chlorine and the quaternary ammonium compounds in 0·1–1% concentrations were most satisfactory. The implications of these investigations, to the fish industry in particular and to the cleanability of wooden surfaces in general, are discussed.

IT IS NOW generally accepted that the spoilage of wet white fish is primarily bacterial, and consequently that an improvement in the standard of sanitation involved in the handling of the fish, besides being of importance from the public health aspect, should result in its being kept in an edible condition for a longer period. The work of Lumley, Piqué & Reay (1929), and later of Reay & Shewan (1949), has shown that improvements in handling, e.g. attention to cleanliness on board the trawler, do lead to marked improvements, up to 30–40%, in the quality of the fish on landing.

Despite these and other recommendations that the various surfaces, particularly boxes, with which the fish might come into contact should be clean (Reay & Shewan, 1939; Wood, 1940), comparatively little work has been done on the application of detergents and disinfectants in the wet fish industry. Bedford (1935) advocated that fishing vessels, after thorough cleansing with water, should be disinfected by means of a 6% formalin solution applied with a high pressure spray. Dunn (1947) gave general principles for the use of detergents and germicides in the fish industry but, due to the paucity of experimental work, could not deal with particular applications. The first real study of the use of disinfectants in the fish industry was made by Tarr (1945, 1947) who investigated the effectiveness of a number of disinfectants—sodium hypochlorite, chloramine-T, cationic detergents, chloro- and phenylphenols and others—in killing vegetative bacterial cells in dilute fish slime, on dry, artificially contaminated wooden surfaces, and in nutrient broth. He found that whereas hypochlorite was the best all round disinfectant, its corrosive action on metals was a problem. The cationic germicides were next best, readily destroying vegetative cells but not spores. Kaylor (1949) recommended chlorine and hypochlorite for keeping bacterial counts on equipment low, and also the use of the sulphonic acid and sulphuric ester detergents for cleaning. The use of chlorination of the water supply for general sanitation in fish plants has also been dealt with (Hurley, 1949).

More recently, some considerable attention has been paid to the sanitation of fish boxes as it has been realized that these are generally the first source of incidental contamination on land. Spilde (1950) recommended to the Norwegian canning industry that herring boxes should be disinfected daily by a preliminary washing with water, followed by immersion for half a minute in a 2–5% sodium hypochlorite solution. Kietzmann (1952) investigated the value of Tego 51, an ampholyte soap, for cleaning fish boxes and found that approximately 97% of the bacteria in the wood were killed when the boxes were washed in water and then sprayed with a 1% solution. Riemann (1953) compared Tego 51 with two cationic detergents and sodium hypochlorite, using artificially contaminated pieces of wood, and found that sodium hypochlorite in concentrations of 0·4–3% was far superior to the other disinfectants, whose behaviour was little different, under his experimental conditions, from that of water.

Previous work (Spencer, 1959) having shown that wooden fish boxes cannot be satisfactorily cleaned by water alone, even with vigorous scrubbing, the use of sanitizers of some sort appeared necessary if satisfactory cleaning was to be obtained. This paper describes an investigation of their use.

METHODS AND MATERIALS

Fish boxes: sampling and cleaning. The boxes, and the method of sampling by scraping, were as previously described (Spencer, 1959) except that only Petri dish counts were made, using heart digest tap water agar. Each count recorded is based on the average of duplicate plates. The method of cleaning was either direct washing by hosing 4 l. of solution into the box with a stirrup pump, or scrubbing with a bass broom during the normal washing procedure. As samples were always taken from the inside, only the internal surfaces were washed. After washing or scrubbing, all boxes were allowed to drain for 10 min in an upright position before sampling, and were sampled again after standing in a cool shed for 48 hr. This was to simulate commercial conditions where boxes are frequently allowed to stand for a day or two before being used. Two boxes were normally used for each investigation, and some investigations were repeated.

Sanitizers. The selection of seven sanitizers used covered a wide range of types including the pure disinfectants hypochlorite and formaldehyde, quaternary ammonium compounds (QACs) with marked bactericidal and slight surface-active properties, detergents with good surface active properties but no bactericidal powers, and compounded substances with both strong detergent and strong bactericidal properties, based on either hypochlorite or QACs. Their characters were as follows: A was a commercial brand of stabilized hypochlorite said to contain 10–12% of available chlorine; B was a commercial nonionic detergent which could be used with A as a detergent-sanitizer mixture; C, a commercial detergent-sanitizer not yet marketed, was said to contain 5% of available chlorine together with various detergents; D was a commercial sanitizer said to contain over 95% of cetyltrimethylammonium bromide; E was a commercial sanitizer said to be a 50% solution of alkyldimethylbenzylammonium chlorides; F, a commercial detergent-sanitizer said to contain 10% of the active constituent of E and compatible detergents; and G was a commercial sanitizer said to contain approximately 10% (w/v) of formaldehyde.

The concentrations used were either those recommended by the manufacturers or, if no recommendations were given, two concentrations approximating to those which might be used in commercial practice.

EXPERIMENTS AND RESULTS

In the first experiment the boxes were washed with the sanitizer without a preliminary washing with water. Boxes washed with water alone served as controls. The results are shown in Table 1. Particularly noticeable is the narrow range of the counts obtained before cleaning. Most of these are within the range 2×10^7—2×10^8/cm^2, indicating relative uniformity in the amount of soil on the surfaces; thus differences between boxes would not vitiate the comparison of the efficiencies of the various sanitizers.

The previous report (Spencer, 1959) of the low cleaning powers of hosing with water alone were confirmed. The maximum reduction in the bacterial numbers was only 60% and the average reduction around 50%, though sanitizers based on chlorine generally effected a 90–99% reduction when the concentration of available chlorine was 1,000 p/m; however, at 100 p/m of available chlorine they were not materially better than water alone. Sanitizer B, the nonionic detergent, either alone or with hypochlorite solutions, did not enhance cleaning efficiency, although there was some evidence that it exerted a slow bactericidal or bacteriostatic action over the subsequent 48 hr standing as it was the only sanitizer with which the reductions after the 48 hr period were greater than those immediately after cleaning. The QAC sanitizers, D, E and F, when used in concentrations of 1,000 p/m or less, also appeared to be ineffective, though when D was used at 5,000 p/m quite marked reductions were obtained. The formaldehyde-containing sanitizer G appeared to have if anything less action than water alone, despite the fact that chemical analysis of samples similar to those taken for the bacterial counts showed the presence of much more than 1 p/m of residual formaldehyde.

An important feature of the results is that 48 hr after cleaning the count was generally higher than immediately after cleaning, even though the boxes were then superficially dry, confirming that during standing the bacteria tend to multiply rather than die out.

Since hosing, even with sanitizers, did not have any marked cleaning effect unless the sanitizers were in relatively high concentration, the next step was to investigate scrubbing, with water alone and with sanitizers.

The results obtained by scrubbing (Table 2) followed, in general, the same pattern as those obtained by hosing alone. The counts on the dirty boxes were of the same order, and whereas the overall cleaning effect was somewhat greater, the various kinds of sanitizer had the same relative efficiencies as in hosing alone. However, sanitizers D and E effected greater reductions, and this was particularly marked with the latter, the benzalkonium QAC, which was effective at a lower concentration than in hosing. However, sanitizer F, in the dilution used, was less effective than E despite the fact that it contained a higher concentration of the same QAC. The chlorine-containing sanitizers were again only effective at 1,000 p/m of available chlorine and sanitizer G was again ineffective at 1,000 p/m of formaldehyde.

Table 1. *The effect of washing with various sanitizers on the degree of bacterial contamination of the inside surfaces of commercial wooden fish boxes*

Sanitizer(s) used	No. of bacteria (10^6/cm^2 of surface sampled)			Reduction in bacteria (%)	
	Before washing	After washing	48 hr later	On washing	Total, after 48 hr
None (water only)	97	51	50	47	49
	140	77	87	45	39
	66	37	46	44	29
	83	33	45	60	45
A, 0·1% (c. 100 p/m av. Cl_2)	32	13·3	26·7	68	17
	61	26·7	26·7	43	43
	67	19·2	24·3	71	64
	81	30	49	63	40
A, 1% (c. 1,000 p/m av. Cl_2)	59	2·56	9·9	95·7	83·5
	57	0·143	0·39	99·8	99·3
B, 0·1% (nonionic detergent)	83	67	21·3	19·3	73
	147	67	26·7	45	83
	15	2·53	0·91	84	94
	104	160	0·92	−50	99
A, 0·1%+B, 0·1% (c. 100 p/m av. Cl_2+ nonionic detergent)	86	18·7	8·3	78	90
	57	2·67	3·73	95·4	94
	92	39·5	69	57	25
	42	12	7·3	71	83
A, 1%+B, 0·1% (c. 1,000 p/m av. Cl_2+ nonionic detergent)	50	4	32	92	37
	187	4·7	5·4	97·5	97
C, 0·2% (100 p/m av. Cl_2)	38	27·7	59	27	−56
	147	29·3	85	80	42
C, 2% (1,000 p/m av. Cl_2)	47·5	1·7	12	96·4	75
	1	0·061	0·09	94	91
D, 0·1% (c. 1,000 p/m QAC)	51	11·8	77	77	−52
	35·5	23·7	13·6	33	62
D, 0·5% (c. 5,000 p/m QAC)	113	0·002	0·093	99·97	99·92
	187	0·56	59	99·7	69
E, 0·04% (c. 200 p/m QAC)	65	43	69	33	−8
	53	24	31·5	55	41
E, 0·2% (c. 1,000 p/m QAC)	200	109	102	47	49
	69	6·5	11·5	91	84
F, 1:160 (c. 630 p/m QAC +detergents)	200	187	160	7	20
	59	24·5	69	58	−17
G, 0·1% (c. 100 p/m formaldehyde)	87	87·5	127	0	−45
	32	37·6	87	−17	−172
G, 1% (c. 1,000 p/m formaldehyde)	22·7	26	42·4	−15	−87
	27·7	38	43·7	−37	−59

Superficially, the boxes after scrubbing appeared much cleaner, detritus and loose slime having been removed, although when sampled considerable amounts of thick glutinous slime were still obtained. The fact that scrubbing was only slightly more effective than hosing alone, when measured by bacterial counts, does confirm that the heaviest contamination is close to the surface of the wood or even within its pores (Wood, 1939; Riemann, 1953; Spencer, 1959).

Table 2. *The effect of scrubbing with various sanitizers on the degree of bacterial contamination of the inside surfaces of commercial wooden fish boxes*

Sanitizer(s) used	No. of bacteria (10^6/cm^2 of surface sampled)			Reduction in bacteria (%)	
	Before scrubbing	After scrubbing	48 hr later	On scrubbing	Total, after 48 hr
None (water only)	1·5	0·3	0·75	80	50
	40·5	20	20·5	51	50
	160	68	52	57	68
	62	40	41	35	35
	1*	0·002	1·73	99·8	−73
A, 0·1% (*c*. 100 p/m av. Cl_2)	63·5	27·7	20	57	69
	37	5·1	17·6	87	53
A, 1% (*c*. 1,000 p/m av. Cl_2)	12·3*	0·037	0·27	99·7	98
	81	23	26·7	71	67
B, 0·1% (nonionic detergent)	54	28·3	27	48	50
	115	62	71	47	38
A, 0·1%+B, 0·1% (*c*. 100 p/m av. Cl_2+ nonionic detergent)	140	69	146	50	−4
	89	11·5	37	87	59
A, 1%+B, 0·1% (*c*. 1,000 p/m av. Cl_2+ nonionic detergent)	11·5*	0·35	0·485	97	96
	110	2·16	22·4	98	80
C, 0·2% (100 p/m av. Cl_2)	155	148	200	5·5	−29
	267	173	160	35	40
C, 1% (500 p/m av. Cl_2)	98	15·5	23·2	87	76
	69	26·7	26	60	60
D, 0·1% (*c*. 1,000 p/m QAC)	61	1·57	7·3	97·5	88
	39·5	31	3·65	92	91
D, 0·2% (*c*. 2,000 p/m QAC)	160	3·7	0·65	98	99·6
	41	0·44	6·7	99	83
E, 0·1% (*c*. 500 p/m QAC)	45	0·022	2·45	99·95	95
	53	1·65	56	96·9	−4
F, 1:160 (*c*. 630 p/m QAC+ detergent)	60	27·2	22	66	62
	46	24	10·4	47	77
G, 1% (*c*. 1,000 p/m formaldehyde)	184	30	16·8	64	91
	148	33·6	29·3	78	80

* Plastic coated boxes.

By chance during this experiment, three markedly clean boxes with hard, non-porous surfaces were secured. These, when new, had been coated with a plastic material as a possible aid to cleanability. It could not be discovered how long these particular boxes had been in circulation, but they were both visibly and bacteriologically much less dirty than the uncoated boxes. Also, they were much more effectively cleaned by scrubbing, even with water alone. The net result was that the counts, both immediately after cleaning and 48 hr later, were much reduced in comparison with those from uncoated boxes.

The low cleaning efficiency of the bactericidal sanitizers, and particularly of the chlorine-containing types, was possibly due to the large amounts of organic material, such as fish slime, present during cleaning. Consequently, the effect of washing the boxes with bactericidal sanitizers after first scrubbing with water alone was investigated, and the results are shown in Table 3. Once again the counts after the preliminary scrubbing were of the same order as those previously obtained after scrubbing with water alone (Table 2), but the cleaning efficiency of the sanitizers was not enhanced by the preliminary scrubbing. This again indicates that the contamination on the box was greatest close to the wooden surface, or even within the wood.

Table 3. *The effect of washing with various sanitizers, after a preliminary scrubbing with water alone, on the degree of bacterial contamination of the inside surfaces of commercial wooden fish boxes*

Sanitizer(s) used	No. of bacteria ($10^6/\text{cm}^2$ of surface sampled)			Reduction in bacteria (%)	
	Before washing	After washing	48 hr later	On washing	Total, after 48 hr
A, 0·1% (*c.* 100 p/m av. Cl_2)	36	26	61	29	−68
	16·8	6·5	30·4	62	−80
A, 1% (*c.* 1,000 p/m av. Cl_2)	55	0·024	2·26	99·95	96
	24·5	12·4	37	50	−52
D, 0·1% (*c.* 1,000 p/m QAC)	7·1	0·088	1·36	98·9	81
	19	4·16	3·65	78	81
E, 0·1% (500 p/m QAC)	17·3	0·38	8·2	98	53
	22	1·47	5·8	93	74
G, 0·1% (*c.* 100 p/m formaldehyde)	29·3	53	52	−80	−76
	44·5	24	26·3	46	55
G, 1% (*c.* 1,000 p/m formaldehyde)	15	11·7	16	21	−7
	53	31·5	82	41	−54

DISCUSSION

The fish boxes used, although extremely dirty, as evidenced by the very high counts obtained before cleaning, were completely representative of the boxes of this type used by the fish trade, having been obtained at random from a commercial establishment on the day they were examined. The present study confirms the results of previous work, and in particular shows that with such boxes it is extremely difficult, if not impossible, to attain a high level of cleanliness. This is not only because of the extreme dirtiness of the boxes, possibly the result of insufficient cleaning in the past, but also because the box material is porous and, due to continuous wetting, also usually soft. The use of such boxes must be deprecated, not only because of the risk of enhanced spoilage of the fish (Spencer, 1959), but also on aesthetic and public health grounds. However, these wooden boxes are likely to remain in use for some considerable time and they must be cleaned as well as possible. To this end, the use of a jet of water alone, or even scrubbing with water, is practically useless. A good disinfectant is necessary, and hypochlorite solutions containing at least 1,000 p/m of available chlorine or quaternary ammonium compounds in suitable concentrations would appear to be reasonably satisfactory. The detergents examined did not appear to enhance the cleaning effect of either water or disinfectant solutions.

It is possible that thorough cleaning by scrubbing and the use of a suitable disinfectant from the first may retard the build up of bacteria on the boxes; but as the wood decays and becomes more porous there would probably be a slow build up under any but perfect cleaning conditions. It does appear that a plastic coating might prevent this by sealing the pores and also make the box more easily cleaned.

How far the results obtained here with wooden fish boxes are applicable to wooden surfaces generally is not known; but these results do confirm those obtained by other workers in both laboratory and field experiments (Tarr, 1947; Spilde, 1950; Riemann, 1953) and it is thought that the problems of cleaning wooden surfaces in general are similar, and that the results obtained here would be applicable to other fields where wooden surfaces have to be cleaned. This would be particularly so where the type of contamination is the same, such as on bulkheads and pound-boards on trawlers, and on filleting benches and troughs on land, where the importance of the contamination of the fish from dirty surfaces is probably greater.

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Clegg, L. F. L., Hoy, W. A. & Suffolk, S. F. (1960). *J. appl. Bact.* **23** (1), 18–20.

AN APPARATUS FOR THE AUTOMATIC DETERMINATION OF CLOT-ON-BOILING END POINT IN RAW MILK

By L. F. L. CLEGG, W. A. HOY and S. F. SUFFOLK
National Institute for Research in Dairying,
University of Reading

Following the reports of the Resazurin Research Committee (Rowlands *et al.* 1950; Eddison *et al.* 1951; Rowlands & Hosking, 1951) and the conclusion that the clot-on-boiling test was the most objective method available for determining the keeping quality of raw milk, Barkworth & Neale (1951) published an addendum describing an apparatus which automatically determined the clot-on-boiling (COB) end point, for use outside normal laboratory working hours. This apparatus, based on a time switch and a solenoid, lifted a basket of test tubes, at any pre-determined time, out of a temperature controlled water bath and placed it in a bath of boiling water. The apparatus allowed only one test to be made at a time. Further, as the functional part contained moving pieces of equipment, the possibility of mechanical failure could not be excluded.

It is clear that the testing for COB end point at regular intervals (the principle used in the Barkworth & Neale apparatus) is a reliable method for determining the keeping quality of milk. Accordingly, an apparatus was constructed which was capable of determining the COB end point at four consecutive three-hourly intervals.

Description of Apparatus

The apparatus is represented in Fig. 1 and consists of a large water bath (A) fitted with an electric immersion heater and thermostat (B). The water is circulated by means of a pump (C). Part of the water from the pump is piped to four outlets (D), each placed over one of four small baths (1–4) which contain the sample tubes. Each outlet is provided with a solenoid-operated valve (E) fitted with a weighted catch (F) so that, once operated, the valve remains closed even when no current flows in the solenoid. The four baths are provided with standing overflow pipes (G) to maintain a constant water level during incubation; the overflow water returns to the main bath. The four baths are fitted with immersion heaters (H) large enough to raise the temperature of the water to boiling point in 10 min. A simple process timer (J) consisting of a synchronous electric motor and cam operated micro-switches completes the unit. A readily visible thermometer should be fitted to the main tank.

Method of Use

The cycle of operation is as follows: with the thermostat bath (A) and circulating pump running, replicate samples of milk in tubes are placed in each of the four small baths 1–4, and the timer started.

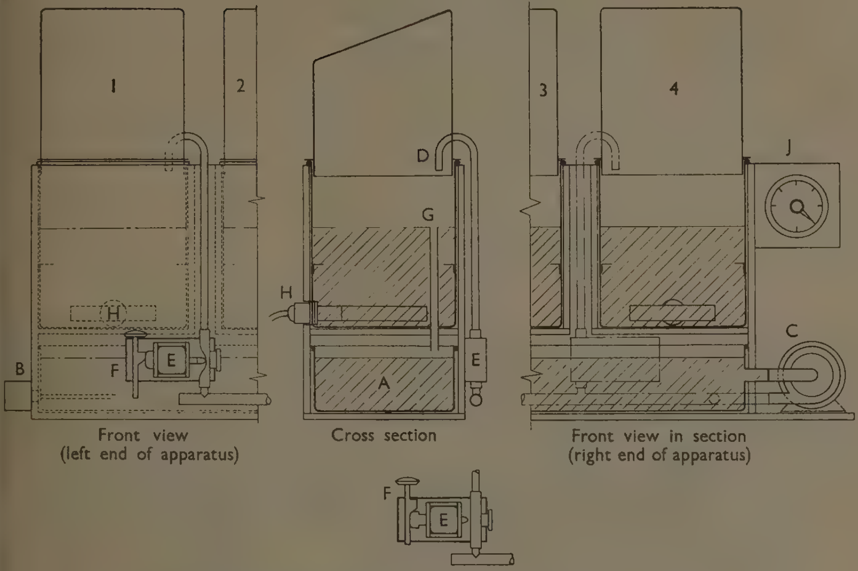

Fig. 1. Diagrammatic representation of apparatus for automatic determination of clot-on-boiling end point.
A, large main water bath; B, immersion heater; C, water pump; D, water flow to small baths; E, solenoid operated valve; F, weighted catch; G, overflow to main bath; H, immersion heater for boiling small baths; J, timing clock.

After three hours the solenoid valve of bath 1 is automatically closed and the immersion heater switched on. After the bath has boiled for about 10 min, the heater cuts off. The solenoid valve remains de-energized but the valve remains closed by reason of the catch (F) and the bath slowly cools to room temperature.

After 6, 9 and 12 hr, similar cycles of operation are carried out with baths 2, 3 and 4, respectively. When all the cycles have been completed the cam on the clock reaches a final micro-switch and then switches off.

Plate 1 illustrates the apparatus with sample tubes in position in racks in the small baths, the lids of which have been opened. Two of the solenoid switch boxes have been opened and the first is shown in the cut-off position. Water is supplied continuously to the main tank from a tap (X) and the overflow is piped to waste (XX).

DISCUSSION

This apparatus has been in use for five years, with entirely satisfactory results. It has been found that, when keeping quality tests are being determined at 22°, a small flow of cool water is necessary to maintain the desired temperature in the main bath during very warm weather and while boiling in the small baths is in progress.

No false positive results, due to germination of spores in the boiled but uncoagulated milk, have been experienced.

It is undesirable to prepare replicate subsamples from fresh milk as it has been shown that this procedure can give rise to inconsistent results because of the uneven distribution of the micro-organisms in the fresh milk. This can be overcome by ageing fresh milk (for about 24 hr) before making replicate subsamples. Except for experimental purposes, it is seldom necessary to test for COB end point, milk which is less than 24 hr old, providing the storage temperature does not exceed 22°.

The present address of Professor L. F. L. Clegg is the Department of Dairy Science, University of Alberta, Edmonton, Alberta, Canada.

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EXPLANATION OF PLATE

PLATE 1. Apparatus for automatic determination of clot-on-boiling end point.

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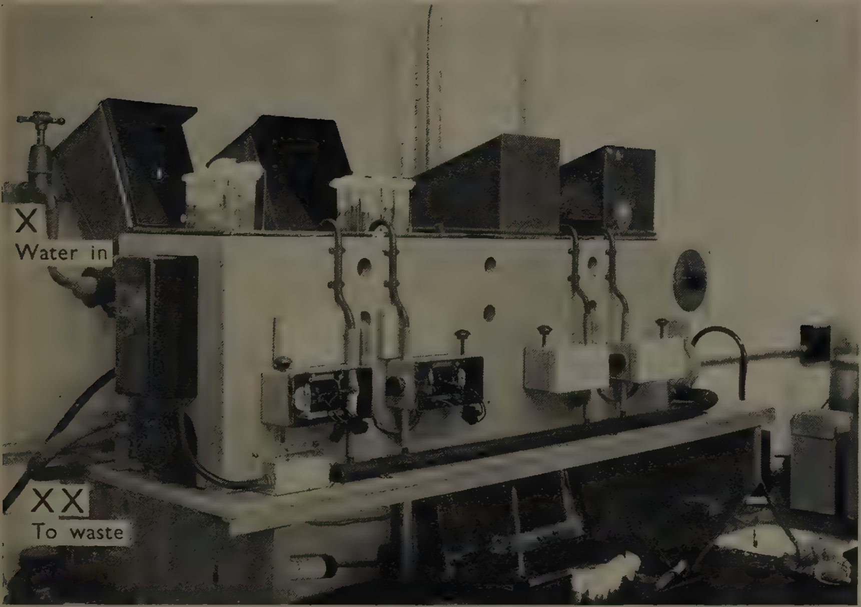
X
Water in
XX
To waste

Clegg, L. F. L., Hoy, W. A. & Suffolk, S. F. — An Apparatus for the Automatic Determination of Clot-on-Boiling End Point in Raw Milk. Plate 1

Knox, R., Penikett, E. J. K. & Duncan, M. E. (1959). *J. appl. Bact.* **23** (1), 21–27.

THE AVOIDANCE OF EXCESSIVE SUPERHEATING DURING STEAM STERILIZATION OF DRESSINGS

By R. KNOX, E. J. K. PENIKETT and MARY E. DUNCAN

Department of Bacteriology,
*Guy's Hospital Medical School, London, S.E.*1

SUMMARY: During the steam sterilization of dressings some degree of superheating occurs. With dressings normally hydrated, that is with about 5% moisture content, the small degree of superheating which occurs is unlikely to be of consequence; but if dressings contain less than 1% of moisture before sterilization, a significant degree of superheating may occur. The use of drums is neither an advantage nor a disadvantage in regulating superheating, which occurs to the same degree whether the air is removed initially by vacuum pump or by downward displacement. The use of a high initial vacuum does not increase the risk of superheating.

During steam sterilization superheating of dressings may occur (Savage, 1937; Walter, 1948; Perkins, 1956). This may result in a decrease in the life of fabrics and, under certain circumstances, in a failure in sterilization.

Superheating can come about in several different ways, either directly or indirectly. It may occur directly when in some way steam that was saturated has been changed into a gas by additional heat. The commonest and most important source of this heat is a sterilizer jacket filled with steam at a higher temperature than that in the chamber. A less common and less important source is found in the reducing valves. Usually reducing valves are far enough from the chamber for the superheat occurring as a result of the reduction in pressure to be dissipated from the surface of unlagged pipes. Furthermore, steam in the main lines is often wet and the liquid phase can, by evaporation, absorb a considerable quantity of sensible heat, usually more than is released by pressure reduction. However, when a reducing valve is close to the chamber to which it is connected by well lagged pipes, and the reduction of pressure is large, the risk of superheating exists, although even under these circumstances this will only be significant when the steam is flowing freely and not when it is flowing intermittently through a thermostatic trap in the discharge line.

Indirectly, superheating of dressings may occur due to the heat generated when water is absorbed by cellulose fibres. It is with this source of superheating that we are concerned here. Walter (1948) drew the attention of the medical world to the fact that superheating due to the wetting of cellulose was related to the initial moisture content of the fibres. The greatest superheating occurs when fibres are initially dry and the least when fibres are initially wet.

Walter (1948) conducted experiments in a gravity displacement sterilizer in which he was able to show large amounts of superheating, as much as 36°, and reported dressings catching fire when the door of the sterilizer was opened. In these experiments dressings of different moisture content were preheated for several hours by a jacket filled with steam, and the applicability of these findings to current practice is doubtful because it is difficult to imagine circumstances under which dressings need to be preheated for more than a few minutes. Walter's experiments were conducted in a gravity displacement sterilizer; but he made the comment that superheating might occur when an initial partial vacuum was used, due to desiccation of fibres and preheating during this operation. Although the practice of drawing a partial vacuum is to be condemned because the subsequent steam penetration time is erratic and unpredictable (Knox & Penikett, 1958) it is not our experience that any significant desiccation is likely to occur during the time taken by an efficient steam ejector to draw the maximum vacuum of which it is capable. It seems likely that many new sterilizers will be fitted with high vacuum pumps for the removal of air from the chamber before steam is admitted (Bowie, 1957), and it is therefore important to know what degree of superheating may occur in these machines.

In these new machines the initial removal of air is so rapid that preheating is unlikely to occur. Superheating due to the jacket being maintained at higher temperatures than the chamber can be avoided by proper design, and the same can be said of the slight risk from reducing valves. The remaining problem is to determine the degree of superheating due to the wetting of cellulose fibres. Urquhart & Williams (1924) determined the equilibrium moisture content of cotton in various conditions, but the temperature range of their experiments was below that used for dressing sterilization. Henry (1959) extrapolated their data and predicted the amount of superheating of soda-boiled cotton to be expected when it was sterilized with steam at 20 lb/in^2. We have compared the degree of superheating at similar pressure occurring after initial high vacuum with that following gravity displacement of air, and we have also compared our experimental results with those predicted by Henry.

MATERIALS AND METHODS

The sterilizer has been described previously (Knox & Penikett, 1958). Dressings of known dry weight were allowed to take up moisture to different degrees. A moisture content of less than 1% was regarded as dry, moisture contents of between 3% and 5% as normal, and moisture contents above 10% as wet. The dressings were loaded into the sterilizer as packs or in drums. Air was removed by gravity displacement or by high vacuum. In the high vacuum experiments the chamber pressure was reduced to 20 mm Hg (absolute) before the admission of steam. In the gravity displacement experiments the removal of the air-steam mixture was controlled by a balanced thermostatic trap. The moisture content of the dressings was determined by a weighing method which, although somewhat inaccurate under these conditions (see Penikett, Rowe & Robson, 1958), was good enough to assign each pack to one of the groups, dry, normal, or wet. The dry weight of the dressings was obtained by drying them under vacuum in a chamber with a jacket heated by steam at 5 lb/in^2 until no further reduction in weight could be obtained. The time taken varied with the size and density of the pack and its initial wetness. A well ventilated load of normal moisture content might be completely dried in 2–3 hr, and would show no further reduction of weight, nor any charring even if left in the evacuated heated chamber for 72 hr. Thus, for convenience in the determination of dry weights, dressings were left in the chamber overnight.

Temperatures were measured with copper-constantan thermocouples. In each experiment four thermocouples were used. Three were stitched into a fold in the fabric, one in the centre of the drum or pack, one at the side and one at the top. A fourth thermocouple was left free in the chamber. The leads from the thermocouples were brought out of the chamber through the hollow pins of a radio valve base. The rim of the valve base made a steam tight connection with a seating fashioned at the end of a tube welded into the chamber. A rubber O-ring was placed between the rim of the valve base and the seating, the valve base being pushed tight against the ring by means of a flanged collar which screwed down on a thread on the outside of the tube. The leads of the thermocouples, which were continuous through the hollow pins of the valve base, were secured in position with solder. The spaces between the pins on the chamber side of the valve base were filled with Bakelite cement to prevent breakdown of insulation by steam. This arrangement permitted easy removal of the whole thermocouple assembly by unscrewing the collar.

The thermocouples were calibrated in the usual way with boiling water and ice, but in addition they were calibrated under the conditions of the experiments by making comparisons between them and two previously calibrated recording mercury-in-steel thermometers. Check calibrations were found necessary between every two or three experiments. The temperatures obtained by the thermocouples and recorded in the results are considered accurate to ±1°. Potentials were measured with a Pye Portable Potentiometer (Cat. No. 7569P).

Each experiment produced results from which it was possible to plot the temperature of the steam in the chamber and the temperatures at the centre, the side and the top of a pack. The experiments were not continued beyond the point where a maximum difference between the steam temperature and the temperature of the load had been reached. When experiments in each group were repeated, the thermocouples were changed in position.

RESULTS

As would be expected, the temperature rises were slower when air was removed by gravity displacement than when it was removed by high vacuum but, disregarding rate, the same pattern of results was obtained. Invariably, the temperature at the centre of a pack of dressings rose above that of the surrounding steam. This was true, though to a somewhat smaller degree, of the temperatures at the sides and tops of packs of dressings in drums. The amount by which the temperature of the dressings rose above that of the steam varied with the initial moisture content. For dry dressings the average superheating was 7·4° while for wet dressings it was only 1·3°

Fig. 1 provides a typical illustration of the superheating occurring in an initially dry pack (with less than 1% of moisture) sterilized in a chamber which had been previously evacuated by a high vacuum pump. The steam temperature rose to 126°, corresponding to the temperature of saturated steam at 20 lb/in^2. The centre of the pack was 7° hotter than the steam and the side and top of the pack were also superheated but to a smaller extent. Fig. 2 shows the results obtained for the same load

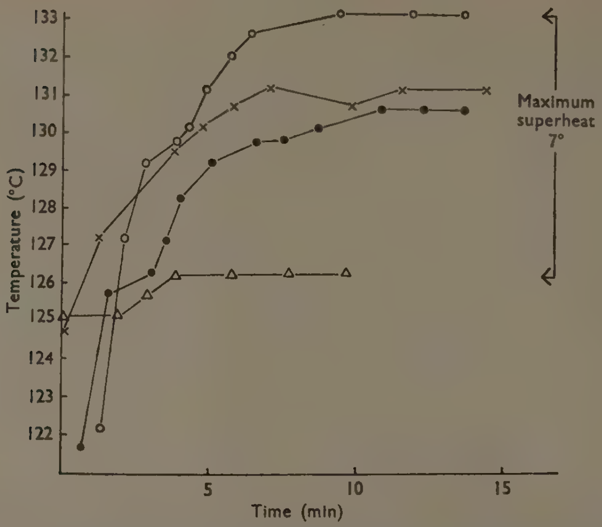

Fig. 1. The changes in temperature at different sites during sterilization of a dressing pack which started unusually dry, i.e. with less than 1% of moisture, and with the air removed by high vacuum pump.
Site in pack: middle, open circles; side, crosses; top, full circles. Triangles, temperature of steam in chamber.

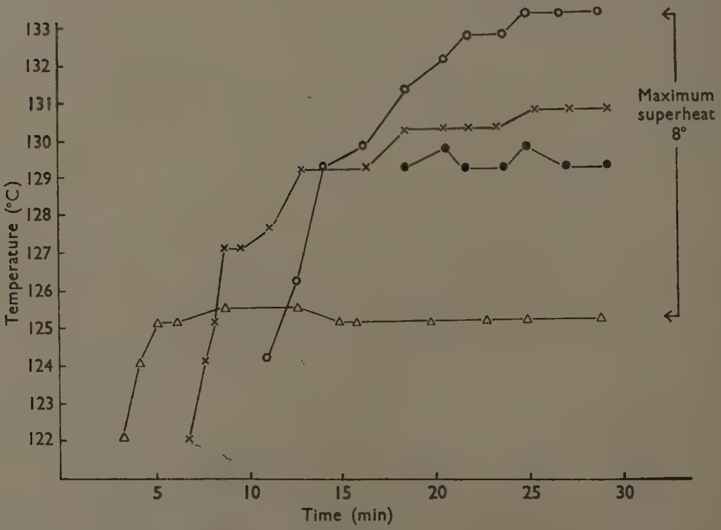

Fig. 2. The changes in temperature at different sites during sterilization of a dressing pack which started unusually dry, i.e. with less than 1% of moisture, and with the air removed by gravity displacement.
Site in pack: middle, open circles; side, crosses; top, full circles. Triangles, temperature of steam in chamber.

sterilized in a chamber evacuated by gravity displacement. Here the temperature at the centre of the pack rose to 8° above that of the surrounding steam. In each experiment the temperature rose rapidly to its highest point and then slowly began to fall. The fall was so slow that usually it was not apparent before sterilization had been completed. This pattern was essentially the same whether the air was removed by gravity displacement or by vacuum pump and was unaffected by the disposition of the dressings in drums or packs.

Table 1. *The effect of moisture content and method of air removal on the superheating of dressings during sterilization*

Initial moisture content (%)	Mean value of superheating (°C.) with air removal by		Grand mean super-heating (°C.)
	High vacuum	Gravity displacement	
<1	7·1 (6 expts.)	7·8 (3 expts.)	7·4
3–5	4·5 (4 expts.)	3·3 (3 expts.)	4·0
>10	1·5 (4 expts.)	1·0 (4 expts.)	1·3

Table 2. *A comparison between the observed values of superheating and those predicted by Henry* (1959)

Initial moisture content (%)	Extent of superheating (°C.)	
	Mean observed values	Predicted values
<1	7·4	9
3–5	4·0	2
>10	1·3	Small*

* Probably negligible.

Table 1 shows the average superheating occurring with dressings of different initial moisture content both with gravity displacement and with vacuum removal of air. In these experiments the dressings were contained in drums, but similar results were obtained in a smaller number of experiments with dressings in packs. The figures are based on the highest values indicated in each experiment by any one of the thermocouples. This was usually the thermocouple which was placed in the centre of the load.

Table 2 gives a comparison of the results obtained in these experiments with the predictions made by Henry (1959). With allowance for the likely experimental error, the agreement is close.

Discussion

The results show that superheating does occur in sterilization procedures and that if dressings start dry, that is, containing 1% or less of moisture, this superheating may be as high as 8–9°. Before considering how this superheating may be avoided it is desirable to consider two possible consequences, damage to fabrics and the effect on sterilization. It is commonly believed that superheating increases the rate at which dressings lose strength, but full data on this point are lacking. There is some evidence to suggest that a more important cause of spoiled dressings is the use of cotton which has been overbleached and thus rendered unusually susceptible to damage by heat (Henry, 1959). The effect of this degree of superheating on sterilization is more important, since the killing of spores is related to the relative humidity to which they are exposed. Savage (1937) found that spores were killed under the conditions of our experiments, that is, with steam at 126° in the chamber but superheated by 10°. Spores were not always killed when the same degree of superheating occurred at lower saturated steam temperatures.

To put dressings into the sterilizer dry is probably undesirable for two reasons. First, the life of textiles may be decreased, and second, there is a risk that sterilization will fail due to the superheating that will occur when the dry dressings take up moisture. The degree of superheating as predicted by Henry and confirmed by us, though not large, should be avoided if possible.

A solution to the problem might be to sterilize dressings in the wet state. If this were done, the life of dressings might be prolonged and sterilization would be certain if the times and temperatures recommended for saturated steam were used. There is, however, a disadvantage in adopting this remedy. Surgeons and theatre staffs require their gowns and towels dry and ready for use. The dryness of the end product of steam sterilization is closely related to the initial moisture content (Penikett Rowe & Robson, 1958), and therefore if dressings are wet when they are put into the sterilizer they will be wet when they come out unless a long drying period is used. A dressing which is subjectively dry contains about 5% of moisture (Penikett *et al.* 1958). At this initial moisture content the amount of superheating (3–4°) is such that the possible depreciation of dressings and the risk of failure of sterilization are insignificant. This moisture content will be attained by dressings which are brought to equilibrium with relative humidities of 45–50%. In the United Kingdom the relative humidity of hospital buildings is very low as central heating is used, but the measurement of relative humidity is easy and precautions should be taken if dressings are likely to be too dry. They should be stored in a cool place.

The results presented show that there is little difference between the superheating which occurs with the gravity displacement and vacuum methods of air removal and there is certainly nothing to indicate that high vacuum is a particular cause of superheating.

We are greatly indebted to Dr. P. S. H. Henry of the Shirley Institute, Manchester, for valuable help and criticisms, and to the Hospital Engineer and his staff, in particular Mr. Ben Winall, for fitting and maintaining the apparatus.

To Messrs. Edwards High Vacuum we owe grateful thanks for the use of their pump and technical advice over the last year; and also to Messrs. Norman Engineering, who have recently lent us a pump which we used in some experiments.

The present address of E. J. K. Penikett is the University of Alberta, Edmonton, Alberta.

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BADGER, E. H. M. & PANKHURST, E. S. (1960). *J. appl. Bact.* **23** (1), 28–36.

EXPERIMENTS ON THE ACCURACY OF SURFACE DROP BACTERIAL COUNTS

BY E. H. M. BADGER AND EILEEN S. PANKHURST
London Research Station, The Gas Council

SUMMARY: A statistical experiment has shown that no significant proportion of the errors of determination of bacterial populations by the surface drop method could be attributed to the dropping technique employed. With both pure and mixed cultures the numbers of colonies/drop followed a Poisson distribution very closely.

It is suggested that in order to determine bacterial populations at a consistent level of accuracy a constant number of colonies should be counted rather than a constant number of drops. Whenever it is justified, the confidence limits for the results of counts should be quoted.

IN THE COURSE of investigations on the biological oxidation of phenols and thiosulphate in gas work effluents the mixed bacterial populations under various conditions of working the plants have been estimated by means of a surface drop method based on that of Miles & Misra (1938). It was desired to know the accuracy of counting in order to determine whether any apparent changes in population were really significant, and the results of investigations into the accuracy of counting these mixed populations, and also of counting pure strains isolated from the mixtures for experiments on growth rate, oxygen uptake, etc., are reported below.

METHOD

The method used for counting pure cultures followed that of Miles & Misra (1938) except that the number of drops plated varied, and the method for mixed cultures differed slightly, since to make the decimal dilutions 1 ml of culture was mixed with 9 ml of diluent. The time required to dry the plates varied considerably from medium to medium.

For each dilution of mixed culture a test tube containing 9 ml of deionized water was used. The 9 ml volume was measured with a Grade B 10 ml pipette, delivering from the 10 ml mark to the 1 ml mark with the tip of the pipette against the side of the tube. The 1 ml samples were delivered by Grade B 1 ml blow out pipettes. Diluent and sample were mixed thoroughly by rolling the tube in an upright position between the hands and by 'flicking' the tube. A separate 1 ml pipette was used for each dilution, and before transfer liquid was sucked up and down at least five times. The test tube was slanted towards the operator and the contents of the pipette were blown out with the side of the pipette resting on the mouth of the test tube and the tip not touching the side of the tube.

For each dilution of pure culture a small sample tube containing 0·9 ml of broth was used. This volume was delivered from the 1·0 ml mark on a Grade B 1 ml pipette to the 0·1 ml mark, with the tip touching the side of the tube. The 0·1 ml of sample was delivered in a similar manner. Mixing was accomplished by 'flicking' the tube and the procedure for transfer was as above, except that the 0·1 ml volume was delivered with the tip of the pipette against the tube.

Pasteur pipettes for delivering drops were drawn in the normal manner and the tips were cut after calibrating in a steel gauge. The pipettes were not calibrated individually for drop volume, but were rotated with the tips at eye level, and those whose tips were seen to be cracked or not cut at right angles to the axis were discarded. Each dilution was drawn up and down the pipette five times, using a rubber teat. The first drop of the final filling was discarded. Drops were delivered by holding the pipette vertically above the plate with the tip approximately 2·0–2·5 cm above the surface of the medium and squeezing the teat gently, allowing a drop to form and fall gently on the medium.

When the drops had dried the plates were incubated at the optimum temperature for growth of the organism or organisms concerned. Mixed cultures were incubated at the temperature of the plants in which they originated, i.e. 30°. The most suitable incubation times were found by experience. Pure strains of phenol oxidizing bacteria were incubated on nutrient agar for 1–2 days or on phenol agar for 2–3 days; mixed cultures oxidizing phenol were incubated on phenol agar for 2–3 days and mixed cultures oxidizing thiosulphate on thiosulphate agar for 5–6 days. An 8× or 10× lens was used to identify very small colonies which sometimes occurred on the mixed culture plates.

EXPERIMENTS

The number of colonies developing usually varies considerably among drops from the same dilution and the degree of variation has to be taken into account in assessing the reliability of the mean value from which the bacterial population is calculated. Variations in the number of colonies/drop may be due not only to random sampling but also to differences in drop size. Comparisons of the results obtained from different plates may also be affected by variation in the media, etc. If an appreciable amount of the variation could be assigned to some specific part of the method of plating the drops, it might be possible to reduce or eliminate it by modifications of the technique. Accordingly an experiment to determine which of the possible causes of variation were significant was planned.

It was decided to test the effects of the use of different pipettes, of variations between drops from the same pipette, of variations between successive fillings of a pipette from the same dilution, and of variations between plates, using mixed cultures from a pilot plant in which the biological oxidation of thiosulphate was proceeding. The experimental design is given in Table 1. Four plates and six pipettes were used and four drops were taken from each pipette from each of two fillings. There were thus 48 drops in all, i.e. 12 per plate. The four sets of 12 drops were similar in that they consisted of 2 drops from each of the six pipettes, six from a first filling and six from a second filling. Each set was also similar with respect to the order of delivery of the drops from the pipettes, i.e. they consisted of four lots of three from the first, second, third and fourth order deliveries. The positions of the drops on the plates were randomized by the use of tables of random numbers.

Table 1. *The experimental design for the determination of the significance of four possible causes of variation in colony counts by the surface drop method*

Plate													
A	Drop	$a_{1,1}$	$a_{1,2}$	$b_{1,1}$	$b_{1,3}$	$c_{1,1}$	$c_{1,4}$	$d_{2,3}$	$d_{2,4}$	$e_{2,2}$	$e_{2,4}$	$f_{2,2}$	$f_{2,3}$
	Position	3	12	11	5	7	9	1	8	10	4	6	2
B	Drop	$a_{1,3}$	$a_{1,4}$	$b_{2,1}$	$b_{2,3}$	$c_{2,1}$	$c_{2,4}$	$d_{2,1}$	$d_{2,2}$	$e_{1,2}$	$e_{1,4}$	$f_{1,2}$	$f_{1,3}$
	Position	9	6	5	4	7	2	3	8	10	11	1	2
C	Drop	$a_{2,1}$	$a_{2,2}$	$b_{1,2}$	$b_{1,4}$	$c_{2,2}$	$c_{2,3}$	$d_{1,3}$	$d_{1,4}$	$e_{2,1}$	$e_{2,3}$	$f_{1,1}$	$f_{1,4}$
	Position	2	12	5	7	11	4	1	10	9	3	6	8
D	Drop	$a_{2,3}$	$a_{2,4}$	$b_{2,2}$	$b_{2,4}$	$c_{1,2}$	$c_{1,3}$	$d_{1,1}$	$d_{1,2}$	$e_{1,1}$	$e_{1,3}$	$f_{2,1}$	$f_{2,4}$
	Position	10	2	8	6	12	9	4	7	1	3	5	11

The six pipettes are represented by the letters a–f. The first suffix number represents the order of filling (first or second) and the second the order of drop delivery (first to fourth).

The results of a preliminary experiment, and of routine counts on both pure and mixed cultures, with various numbers of replicate drops, have also been analyzed below.

RESULTS

The statistically planned experiment

The 10^{-3} dilution was the most suitable for counting and the colony counts in the 48 drops are given in Table 2. Equal numbers of colonies were obtained from the first and second fillings of the pipettes, viz. 479. This was no doubt fortuitous, but clearly no significant variation could be assigned to differences between fillings. The

Table 2. *The numbers of colonies in the drops from a 10^{-3} dilution of a mixed culture in an experiment designed according to Table 1*

Pipette		First filling					Second filling					Grand total of colonies/ pipette
		Drop order				Total colonies/ pipette	Drop order				Total colonies/ pipette	
		1	2	3	4		1	2	3	4		
a		11	14	22	15	62	17	34	14	17	82	144
b		17	32	14	18	81	25	18	27	20	90	171
c		25	23	24	14	86	12	20	32	19	83	169
d		20	22	24	20	86	17	21	21	24	83	169
e		19	27	15	16	77	16	21	12	24	73	150
f		28	10	25	24	87	17	15	21	15	68	155
Totals	Drop	120	128	124	107		104	129	127	119		
	Filling					479					479	
	Expt.											958

Table 3. *The total numbers of colonies according to plate and order of drop delivery, derived from Table 2*

Plate	Total colonies	Order of drop delivery	Total colonies
A	221	1	224
B	236	2	257
C	277	3	251
D	224	4	226
Grand totals	958		958

pipette totals are given in the right hand column of Table 2 and the drop and plate totals obtained after compounding the results from both fillings are given in Table 3.

The results were then subjected to an analysis of variance with the object of revealing whether any of the observed variation could be ascribed to bias either in the sense that one or more of the pipettes or plates was consistently tending to give high or low results or that there were real differences between the drops according to their order of delivery. The method of calculation is given in statistical text books, e.g. that by Davies (1957), and the summarized results are given in Table 4. None of the mean squares between pipettes, drops or plates was significantly different from that of the 'remainder'. The whole of the observed variation could therefore be ascribed to chance, i.e. to inherent differences among the drops themselves, and there was no superimposed bias. The absence of bias can be demonstrated by assigning the observations to pipettes, drop orders and plates in a random manner. The totals so obtained are given in Table 5, which shows that the variations obtained in this way were as large as when the observations were properly grouped. The experiment showed therefore that no appreciable errors were being introduced by faults in technique. There would be no significant gain in accuracy by attempting a still closer standardization of the method of dropping, e.g. by using specially manufactured metal pipettes instead of glass pipettes prepared in the laboratory, or by calibrating individual pipettes gravimetrically, and it can be concluded that the main cause of difference in colony count from drop to drop is due to variation in the number of viable bacteria present in the drop at the time of inoculation.

Table 4. *A summary of the analysis of variance of the results in Tables 2 and 3*

Source of variation	Sum of squares	Degrees of freedom	Mean square
Between pipettes	83	5	16·6
Between drops	72	3	24·0
Between plates	166	3	55·3
Remainder	1,141	36	31·7
Totals	1,462	47	—

Table 5. *The analysis of variance of a random assignment of the colony numbers among pipettes, drops and plates*

	Randomly assigned colony numbers						Sum of squares	Mean square
Pipettes	172	162	138	158	179	149	140	28·0
Drops	242	248	217	251			60	20·0
Plates	234	242	262	220			77	25·7
					Remainder		1,185	32·9

Other experiments: agreement with expectation and accuracy

It has been pointed out that bacterial counts determined by the plating method should theoretically follow a Poisson distribution (Fisher, Thornton & Mackenzie, 1922) although close agreement has not always been found (Wilson *et al.* 1935). A

Poisson distribution may be expected to be observed when the number of individuals that can be selected for counting is very large but the chance that any particular one finds its way into the sample is very small. The counting of particles in gas in the ultramicroscope is an example (Badger, 1946; Davies, 1957) and it also applies to accident rates in factories. When surface drop counts follow a Poisson distribution the frequency of occurrence of 0, 1, 2, ...x... organisms/drop will be given by the terms of the expression $Ne^{-m}\left(1, m, \frac{m^2}{2!}, \dots \frac{m^x}{x!}, \dots\right)$ where N is the total number of organisms counted and m is the mean number of organisms/drop.

For high values of the mean, Poisson distributions are closely similar to normal distributions but at low values of the mean they are markedly skewed. As an illustration of this skewness the colony counts of 95 drops, obtained in a pilot experiment carried out before the one described above, in which the mean number of colonies/drop was 2·6, may be considered. The numbers of drops containing 0, 1, 2, 3 ... colonies are given in Table 6, together with the numbers expected from a Poisson distribution, and it can be seen that the distribution is not symmetrical about the mean. The agreement between the observed and expected values in most of the groups was very close and none of the differences was significant at the 0·05 probability level when the χ^2 test was applied.

Table 6. *A comparison of the observed and expected distributions of colonies in 95 drops*

No. of colonies/drop (x)		0	1	2	3	4	5	6	>6
No. of drops with x colonies	Observed	7	22	26	12	16	5	3	4
	Expected	7·06	18·34	23·84	20·67	13·43	6·99	3·03	1·64
	Difference	0·06	3·66	2·16	8·67	2·57	1·99	0·03	2·36
	χ^2	0·00	0·73	0·20	3·64	0·49	0·57	0·00	3·40

Note: the significance levels for χ^2 are: $P=0{\cdot}05$, $\chi^2=3{\cdot}84$; $P=0{\cdot}01$, $\chi^2=6{\cdot}63$.

An important property of Poisson distributions is that the standard deviation is equal to the square root of the mean. This affords a means of assessing the limiting accuracy of counting, as will be discussed later. But it also provides a method of testing how closely the theoretical distribution has in practice been followed in small sets of drops, such as are used in routine testing. With small sets the number of drops is insufficient to enable the observed and expected distributions of colonies/drop to be compared by the method used above with large numbers. Instead the standard deviations may be compared with the expected values. This has been done in Fig. 1 for 77 routine counts, in which the number of drops was usually 3 or 4 but varied between 2 and 8. Two observers carried out these counts, which were on both pure and mixed cultures, and in order to simplify the mass of data the results have been grouped according to the level of the mean number of colonies/drop at approximately 10 unit intervals. The standard deviations were calculated as a percentage of the mean number/drop and on this basis the values decreased as the mean number/drop increased. It is clear that the theoretical relation was closely followed with no obvious difference between the two observers or between the pure and mixed cultures.

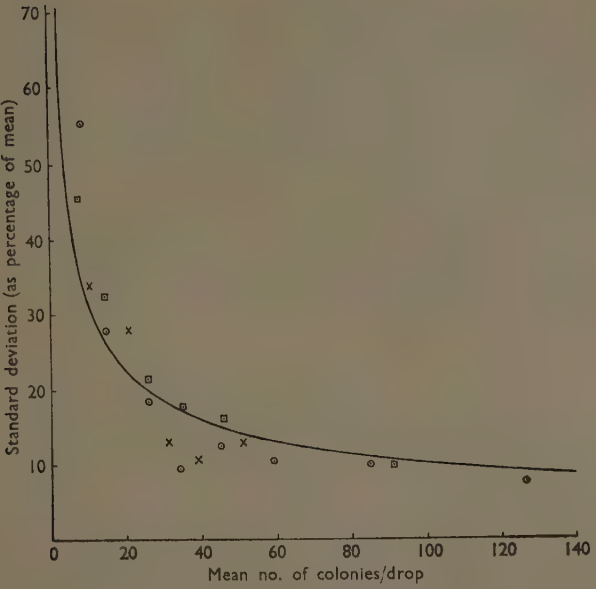

Fig. 1. The relationship between the standard deviation and the mean number of colonies/drop for 77 routine surface drop counts on both pure and mixed cultures. The data have been simplified by grouping them according to the level of the mean number of colonies/drop at approximately 10 unit intervals. Circles, pure cultures, first observer; squares, pure cultures, second observer; crosses, mixed cultures, first observer. Continuous line, theoretical curve (Poisson distribution).

It was pointed out by Fisher *et al.* (1922) that a precise measure of the closeness of fit in Poisson distributions is given by the χ^2 test, using the difference between $\sqrt{2\chi^2}$ and $\sqrt{2\varnothing-1}$ where $\varnothing$ is the number of degrees of freedom, when the number of counts concerned is large (>30). This difference is normally distributed with unit standard deviation. The details for the same data as were used in Fig. 1 are given in Table 7. Here, $\sqrt{2\chi^2}=22{\cdot}63$ and $\sqrt{2\varnothing-1}=22{\cdot}02$, and the difference, 0·61, is small enough to indicate good agreement between the experimental and theoretical values.

Table 7. *Details of the χ^2 test applied to 77 routine colony counts with various numbers of replicate drops*

No. of drops/set	No. of sets	Degrees of freedom	Total χ^2
2	2	2	6·08
3	23	46	34·08
4	41	123	144·69
5	1	4	11·30
6	1	5	2·51
8	9	63	57·42
Totals	77	243	256·08

Table 8. *Details of the χ^2 test applied to routine colony counts from pure and mixed cultures separately*

Type of culture	Degrees of freedom ($\varnothing$)	Total χ^2	$\sqrt{2\chi^2}$	$\sqrt{2\varnothing-1}$	Difference $\sqrt{2\chi^2}-\sqrt{2\varnothing-1}$
Pure	174	181·49	19·05	18·63	0·42
Mixed	69	74·59	12·21	11·70	0·51
Totals	243	256·08			

These counts were also separated into two groups, according to whether they were obtained from pure or mixed cultures, and the χ^2 test applied to each. The details of these tests are shown in Table 8. Evidently there was good agreement with expectation in both cases.

Discussion

When the number of colonies/drop follows a Poisson distribution, as has been shown to be the case in the present work, the accuracy attainable is related to the total number of colonies counted. Consider a set of n drops with a mean number of m colonies/drop and a total of N (=nm) colonies. The standard deviation of single drops in the test is $\sqrt{m}$, or $100/\sqrt{m}$ expressed as a percentage of the mean, and the standard deviation of the mean is $100/\sqrt{(mn)}=100/\sqrt{N}$. The 95% confidence limits are therefore $100t/\sqrt{N}$, where t is the appropriate value of "Student's" function ($t=1{\cdot}96$ for large values of N) and it follows that if the estimate of the bacterial population, obtained from a sample containing N bacteria, is to be within ±a% of the true value on 19 occasions out of 20, the relationship $100t/\sqrt{N}=a$ must hold, i.e. $N=10^4t^2/a^2$. The relation between a and N is illustrated in Table 9 and Fig. 2, from which it can be seen that the accuracy is very low for $N<50$.

Table 9. *The relationship between accuracy (at 95% confidence limits) and the total number of colonies counted, for Poisson distributions*

Accuracy (% of mean)	No. of colonies counted
± 5	1,537
±10	386
±15	173
±20	98
±25	64
±30	45
±35	34

Now the usual procedure when carrying out the drop method is to incubate a constant number of drops and to count all the colonies. The disadvantage of this is that the level of accuracy of the result will depend on the total number of colonies counted, and this is not completely under control. It would be better to endeavour to count a constant number of colonies, the number being determined by the level of accuracy required, rather than a constant number of drops. This can be done by plating out more drops than are likely to be needed and counting colonies only until the count on the drop in which the required number is reached has been completed. For many purposes a total count of about 100, giving an accuracy of ±20% would be adequate. To improve the level of accuracy to ±10% would require 400 colonies. This might involve the use of more than one plate/dilution or, alternatively, varying the dilution by a factor of 5 instead of 10. With the media and organisms used in the present work it was found that twelve 0·025 ml drops could be accommodated on a 3½ in. plate.

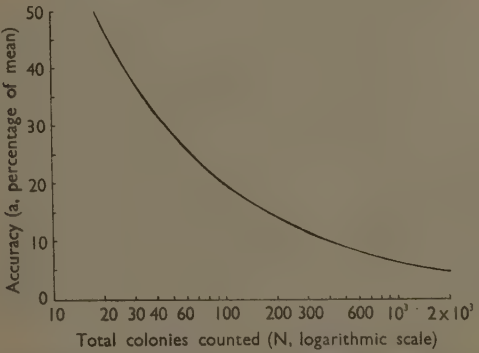

Fig. 2. The relationship between accuracy (at 95% confidence limits) and the total number of colonies counted, for Poisson distributions.

It should be stressed that the levels of accuracy quoted with respect to the total number of colonies apply only to counts which follow a Poisson distribution. If this condition does not hold, the errors are likely to be greater. Miles & Misra (1938) stated that the conditions necessary for realizing a Poisson distribution, enumerated by Fisher *et al.* (1922) for counts of mixed bacterial populations by the poured plate method, were easier to fulfil for surface-viable counts of pure cultures. In the present investigation, using the surface method, we have found good agreement with the Poisson distribution with both pure and mixed cultures. Whenever this can be established, confidence limits for the results of counts should be quoted.

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THE DIFFERENTIATION OF *PSEUDOMONAS* FROM OTHER GRAM-NEGATIVE BACTERIA ON THE BASIS OF ARGININE METABOLISM

By MARGARET J. THORNLEY

Low Temperature Research Station, Cambridge

SUMMARY: Of 391 Gram-negative bacteria isolated from chicken meat spoiled at a low temperature and classified by the commonly used methods, 156 were considered to be *Pseudomonas* and 188 *Achromobacter*, and 47 others belonged to the coli-aerogenes group or remained unclassified. A test for the production of alkaline conditions in an arginine medium incubated under a vaseline seal gave positive results for 155 of the *Pseudomonas* isolates, and negative results for 1 *Pseudomonas* and all the 188 *Achromobacter* strains. When named strains from culture collections were tested under these conditions, 63 *Pseudomonas* strains produced alkalinity while two plant pathogenic *Pseudomonas* species and two non-pigmented strains did not. These last two, which produced no acid from glucose, could not be regarded as typical *Pseudomonas*. All the *Achromobacter* strains gave negative results, as did four *Alcaligenes*, but one species, *Alcaligenes bookeri*, produced slightly alkaline conditions. One strain of *Chromobacterium* and three of *Vibrio* were also positive. These could be distinguished from *Pseudomonas* by their metabolism of glucose.

The micro-organisms which commonly spoil food such as meat, fish, eggs and milk in cold storage are psychrophilic Gram-negative bacteria, and are often referred to as the *Pseudomonas-Achromobacter* group (Mossel & Ingram, 1955). Experience with meats suggests that many of these bacteria have polar flagella but lack ability to form pigment (Brown & Weidemann, 1958) and, as already noted (Ayres, 1955; Brown & Weidemann, 1958) they have been classified as *Achromobacter* or *Pseudomonas* according to the criteria adopted. Early workers limited *Pseudomonas* to strains producing a water soluble green pigment, while non-pigmented motile strains were called *Achromobacter* whether the flagella were polar or peritrichous. This system is still used by many French workers (Brisou, 1957). The definition was changed in the 6th edition of Bergey's Manual of Determinative Bacteriology (Breed, Murray & Hitchens, 1948), where *Pseudomonas* was described as a genus of polarly flagellated straight rods which might or might not produce water soluble pigments, while the organisms in the genus *Achromobacter* were non-pigmented and either non-motile or peritrichous. In the 7th edition of Bergey's Manual (Breed, Murray & Smith, 1957) the previous definitions were retained except that the genus *Pseudomonas* was limited to strains which were known or thought to attack glucose by an oxidative pathway only. In this paper the criteria of Bergey (7th edition) will be adopted when '*Pseudomonas*' and '*Achromobacter*' are referred to. The oxidative attack on glucose, which is characteristic of *Pseudomonas*, is possessed also by some *Achromobacter* strains (Shewan, pers. comm.); but it is useful in helping to distinguish between *Pseudomonas* and members of the Enterobacteriaceae. The oxidase test of Kovacs (1956) was also devised for this purpose, using clinical material, a positive result signifying

Pseudomonas. This observation was confirmed by Shewan for *Pseudomonas* strains isolated from fish. while *Achromobacter* strains from the same source could be either positive or negative. When tested against penicillin. *Pseudomonas* strains were resistant to 2.5 i.u., whereas *Achromobacter* were sensitive (Shewan, Hodgkiss & Liston, 1954). Sensitivity to a vibriostatic agent was used to separate out vibrios.

In these reports the only characters distinguishing *Pseudomonas* from *Achromobacter* are flagellation, if the strains are motile, and penicillin sensitivity. In addition, failure to produce acid from glucose and a negative oxidase test are likely to occur in *Achromobacter* rather than *Pseudomonas*. The difficulties encountered in practice arise from the time consumed in flagella staining, which may render that procedure impracticable for large numbers of isolates and, even when it is used, the problem of classifying non-motile organisms remains. This is well illustrated by the work of Brown & Weidemann (1958).

In the present work Gram-negative bacteria from chicken meat spoiled at low temperature were classified by the tests outlined above, with flagella staining applied only to a selection of strains. A new test was developed and tried out on these bacteria and on named strains from culture collections.

METHODS AND MATERIALS

In the preliminary classification of the cultures the following techniques were used.

Incubation. All tests were done at 20°.

Motility. Overnight cultures in yeast extract broth were examined under low power dark ground illumination.

Glucose metabolism. The mode of action on glucose was studied by means of the semisolid medium of Hugh & Leifson (1953) (see Table 1).

Fluorescence. Cultures were streaked on the medium recommended for maximum fluorescin production by King, Ward & Raney (1954) and examined under ultraviolet light after one day's incubation.

Antibiotic sensitivity. The method of Shewan, Hodgkiss & Liston (1954) was employed with penicillin (2.5 i.u.) and terramycin (10 μg) Sentest tablets (Evans Medical Supplies, Ltd.).

Oxidase test. Kovacs' (1956) method was used, with cultures grown on solid medium for either 24 or 48 hr.

Flagella stain. The modification of Fontana's method described by Rhodes (1958) was used.

During classification by means of these tests it was observed (as will be detailed later) that strains grouped as *Pseudomonas* because they produced no acid from glucose in Hugh & Leifson's medium incubated anaerobically were not inert but shifted the pH towards the alkaline side. This led to a study of the production of alkaline substances from the peptone, from a simplified medium, and from known amino acids in experiments with washed cells. The following methods were used.

Change of reaction in peptone medium. An adaptation of Hugh & Leifson's medium resulted in medium 1, whose composition is shown in Table 1. As will be seen, the carbohydrate was omitted, the peptone concentration was increased and the indicator was changed to phenol red. To obtain the results to be described it was essential to use Difco Bacto-peptone, as three other brands of peptone gave different results. This medium was filled into 25 ml bottles with screw caps and rubber washers in amounts of 15 ml and sterilized by autoclaving.

To perform the test, duplicate bottles were inoculated by stabbing. Sterile melted vaseline was poured into one bottle to a depth of 5 mm; the screw cap of the other bottle was left loose. Changes in reaction, shown by colour changes of the indicator, were noted at intervals during 21 days incubation at 20°.

Table 1. *The relationship between the compositions* of Hugh & Leifson's medium and media* 1, 2 *and* 2A

Ingredient	Composition (% w/v)			
	Hugh & Leifson	Medium 1	Medium 2	Medium 2A
Peptone	0·2	0·5†	0·1	0·1
NaCl	0·5	0·5	0·5	0·5
K_2HPO_4	0·03	0·03	0·03	0·03
Agar	0·3	0·3	0·3	0·3
Bromothymol blue	0·003	—	—	—
Phenol red	—	0·001	0·001	0·001
Carbohydrate	1·0	—	—	—
Arginine HCl	—	—	—	1·0

* The pH of Hugh & Leifson's medium is 7·1, and that of media 1, 2 and 2A was 7·2. †Difco Bacto-peptone must be used.

Change of reaction in arginine medium. The basic medium 2 was similar to medium 1 but had less peptone (Table 1). It was used alone or with the addition of 1% of L(+)-arginine monohydrochloride, when it was called medium 2A. The pH in both cases was adjusted to 7·2. All ingredients were dissolved and autoclaved together in 5 ml bijou bottles which were filled to a depth of about 16 mm. They were inoculated in duplicate and one bottle was sealed with vaseline as with medium 1.

Techniques in experiments with washed suspensions

Washed suspensions were incubated at 25° under strictly anaerobic conditions in Thunberg tubes with various substrates, including peptone solutions and amino acids used singly and combined in groups.

Preparation of suspensions. The organisms were inoculated into 10 ml amounts of medium 1 (without the agar) in T-tubes (Kay & Fildes, 1950) and incubated at 25° with shaking for 2 days. The cells were centrifuged, washed twice in quarter-strength Ringer's solution and resuspended in the same solution to a standard opacity as measured by the EEL nephelometer. These suspensions were stored at 1°. No difference in activity was observed whether they were used the day after preparation or up to a week later.

Substrates. Medium 1 without the agar was compared with amino acids, mainly combined in groups as in the experiments of Hills (1940). Table 2 shows the constituents of the groups with the concentrations at which they were initially present in the reaction mixtures. Phenol red was added to give a final concentration of 0·001%, as in medium 1.

Table 2. *Amino acids, grouped after Hills* (1940), *as used in experiments with washed suspensions*

Group	Composition	Concn. ($M \times 10^{-4}$)
I	Glycine	40
	DL-alanine	40
	L(−)-proline	40
	DL-valine	40
	DL-leucine	40
II	DL-isoleucine	40
	DL-norleucine	40
	L(−)-phenylalanine	40
	L(−)-tyrosine	2
III	L(−)-histidine	40
	DL-tryptophane	40
	DL-lysine†	26
	L(+)-ornithine†	40
	L(+)-citrulline	40
IV	DL-serine	40
	DL-threonine	40
	DL-methionine	40
	L(−)-cystine	Trace
	L-cysteine HCl	Trace
V	DL-aspartic acid	40
	L(+)-glutamic acid	40
	DL-asparagine	40
	L(+)-glutamine	40
Used alone	L(+)-arginine*	40

* Monohydrochloride. † Dihydrochloride.

Method. In a typical experiment, 4·5 ml of the substrate solution were put into each tube and 0·5 ml of the bacterial suspension in the stopper. The tubes were evacuated and filled with oxygen-free nitrogen four times, placed in a water bath at 25°, and the contents mixed. The colour changes were recorded every hour and, when the experiment was stopped, pH measurements were made with a glass electrode.

RESULTS

This work resulted from an investigation of the psychrophilic spoilage flora of chicken carcases which had been treated with ionizing radiation, with chlortetracycline, or had received both treatments. Two experiments were performed, in which samples were taken from control and treated birds initially and after they had spoiled during storage at 1° and 3°, and the bacteria enumerated on heart infusion agar (Difco) plates incubated at 1° and 20°. From these plates colonies were picked, and 391 cultures of Gram-negative bacteria were obtained. The tests used to classify these organisms were those recommended by Shewan (pers. comm.), and on the results obtained they were placed in five groups, A–E, described below. Some of the groups were subdivided, and the characters of the organisms, separated into classes according to their action on glucose, are set out in Table 3.

Group A, *gas-producing fermenters.* These were recognized by their behaviour in the Hugh & Leifson test. They were penicillin resistant, motile or non-motile and, with one exception, oxidase negative. Further study of some of these cultures (marked A_1 in Table 3) showed them to belong to the genera *Aerobacter* (*Cloaca*) and *Hafnia*, while the one oxidase positive strain (marked A_2) was an *Aeromonas* (Eddy & Kitchell, 1959).

Table 3. *The characters and a grouping of* 391 *Gram-negative organisms isolated from chicken carcases*

Test	Reactions* of organisms classed on type of glucose metabolism as:													
	Fermenters, group†			Oxidizers, group†					Inert, group†					
	A_1	A_1	A_2	B	C	E_1	D_2	D_2	D_1	D_2	D_2	D_3	D_4	E_2
Motility	—	+	+	+	+	—	—	—	+	—	—	—	—	+
Fluorescence	—	—	—	+	—	—	—	—	—	—	—	—	—	—
Penicillin	R	R	R	R	R	R	S	S	S	S	S	R	R	R
Oxidase	—	—	+	+	+	+	+	—	+	+	—	+	—	+
No. of isolates Exp. 1	8	17	1	16	11	0	19	0	3	46	18	4	14	7
No. of isolates Exp. 2	4	6	0	31	98	2	8	5	1	27	34	5	6	0
Total isolates	12	23	1	47	109	2	27	5	4	73	52	9	20	7

* +, present; —, absent; R, resistant; S, sensitive.

† Groups: A, gas-producing fermenters of glucose; B, pigmented *Pseudomonas*; C, non-pigmented *Pseudomonas*; D, *Achromobacter*; E, unclassified.

Group B, *pigmented* Pseudomonas. Forty-seven cultures produced fluorescent pigment on the medium of King *et al.* (1954) and also were penicillin resistant and oxidase positive. All were motile rods, straight or slightly curved, and all produced acid oxidatively from glucose.

Group C, *non-pigmented* Pseudomonas. Among the non-fluorescent, penicillin resistant, oxidase positive cultures, 109 were motile and oxidized glucose. Flagella stains were done on about one-fifth of these. They had polar flagella, and were classified as non-pigmented *Pseudomonas*. Two similar but non-motile cultures were left unclassified (group E_1, Table 3).

Group D, Achromobacter. Strains which were sensitive to penicillin and either oxidative or inert with glucose were at first regarded as *Achromobacter*. They were either positive or negative in the oxidase test. Only 4 strains in this group were motile (D_1, Table 3) and 2 of these showed polar flagella, which excluded them from *Achromobacter*. They were finally left unclassified. The flagellation of the other 2 strains could not be determined: a heavy deposit of stain surrounded each cell, probably due to the presence of mucilaginous material. These cultures were provisionally retained in this group. The non-motile penicillin sensitive organisms (D_2. Table 3) showed a tendency for some cells to stain Gram-positive, especially when freshly isolated, and were either coccoid rods, often in pairs, or even cocci, some resembling *Neisseria*. This very characteristic morphology corresponded with that described by Shewan and his colleagues for *Achromobacter* from fish, and it also occurred in two groups of organisms resistant to penicillin and inert towards glucose (D_3 and D_4, Table 3). Group D_3 contained 9 oxidase positive and group D_4 20 oxidase negative strains. Although similar penicillin resistant groups had not been found by Shewan, groups D_3 and D_4 were included in *Achromobacter* on the morphological grounds. Excluding the 2 polarly flagellated penicillin sensitive D_1 cultures mentioned above, this made a total of 188 *Achromobacter* strains. The 2 non-motile glucose oxidizing organisms previously mentioned under group C (E_1, Table 3) did not fit into this category, as they were slender rods of medium length.

Group E, *other organisms*. In addition to the 2 polarly flagellated organisms in group D_1, and the 2 non-motile organisms similar to *Pseudomonas* (group E_1), 7 penicillin resistant, oxidase positive, motile organisms which were inert on glucose remained unclassified (group E_2, Table 3). They formed yellow pigment on milk agar and in gelatin stabs. Flagella stains were done on 4 of these strains and all proved to have a single polar flagellum. They were excluded from *Pseudomonas* because of the failure to produce acid from glucose; from *Flavobacterium* by their polar flagella; and from the genus *Lophomonas* of Galarneault & Leifson (1956) by the fact that they were monotrichous.

As will be seen from this description the borderline between *Achromobacter* and *Pseudomonas* is doubtful, and the separation made here was based mainly on morphological grounds. The remainder of this paper concerns the development of an additional method of distinguishing these two genera.

Change of reaction in peptone medium

It was observed that when glucose oxidizing organisms were incubated in Hugh & Leifson's medium an alkaline reaction sometimes appeared in the closed bottle. This, which seemed to happen mainly with *Pseudomonas* strains, was attributed to the metabolism of peptone, and in order to find out more clearly what was happening tests were done in the modified Hugh & Leifson medium (medium 1, Table 1).

Table 4. *The reactions produced in medium* 1 *by* 380 *Gram-negative bacteria isolated from chicken meat*

Reaction*, in bottle		Group of organisms	Proportion showing reaction
Open	Closed		
Alk.	Alk.	B. Pigmented *Pseudomonas*	46/47
		C. Non-pigmented *Pseudomonas*	109/109
Alk.	NC or slight acid	B. Pigmented *Pseudomonas*	1/47
		D. *Achromobacter*	188/188
		A. Gas producing fermenters	3/36
Alk.	Acid	A. Gas producing fermenters	33/36

* Alk., alkaline reaction; NC, growth with no change in reaction.

Both open and closed bottles of this medium were inoculated, and in the open bottles almost all the organisms produced an alkaline layer near the surface, but at differing rates. The reactions in the deeper layers and in the closed bottles varied, as shown in Table 4. Of the 47 pigmented *Pseudomonas* strains described above, 46 produced alkali in closed bottles and 1 gave no change. The 109 non-pigmented *Pseudomonas* all produced alkali, as did the two similar but non-motile organisms. The 188 organisms classified as *Achromobacter* gave no change in closed bottles, or a very slight shift to the acid side. Three gas-producing fermenters (1 *Aeromonas*, 1 *Hafnia*, 1 not studied in detail) behaved in the same way, while the other 33 gave definite acidity in closed bottles together with strong surface alkali production in open bottles. Thus the *Pseudomonas* and *Achromobacter* strains gave different reactions when incubated in closed bottles of medium 1, and the results were sufficiently promising for further investigation of the nature of the reaction concerned. The conditions in the closed bottles were not strictly anaerobic, since the medium had not been boiled to remove dissolved oxygen and contained no reducing substances. It was in fact shown, by inoculating 2 *Pseudomonas* and 2 *Achromobacter* cultures on to plates of the same medium solidified with 1·5% of agar and incubating in a McIntosh and Fildes jar, that in strictly anaerobic conditions no growth took place, at least with the strains selected. It was therefore possible that the *Pseudomonas* cultures possessed a greater capacity than the *Achromobacter* for growth at low oxygen tensions and hence the difference might be quantitative. To check this possibility some experiments were made with washed suspensions incubated in oxygen-free nitrogen in Thunberg tubes.

Experiments with washed suspensions and peptone

In the first experiment two strains, one of *Pseudomonas* and one of *Achromobacter*, were grown under two sets of conditions: (a) in 10 ml portions of medium 1 (agar omitted) in 25 ml bottles and (b) in 10 ml portions of medium 1 (agar omitted) in T-tubes, with aeration. Washed suspensions in Ringer's solution were prepared and adjusted to approximately the same opacity. When these suspensions were added to medium 1 in Thunberg tubes and incubated under nitrogen the reaction in the tubes containing *Pseudomonas* cells became alkaline, no matter whether they had been grown in aerated or non-aerated conditions. No change in reaction took place with the *Achromobacter* cells. Therefore all later experiments were done with cells grown in aerated culture.

Table 5. *The pH changes induced in medium* 1 *after incubation anaerobically for* 22 *hr with washed suspensions* of* Pseudomonas *and* Achromobacter *cells*

Culture		pH changes in duplicate tubes	
Group	no.		
B. Pigmented *Pseudomonas*	140	+0·79	+0·70
	145A	+0·31	+0·28
C. Non-pigmented *Pseudomonas*	155A	+0·72	+0·73
	167	+0·64	+0·64
D. *Achromobacter*	111	−0·03	+0·01
	147	−0·04	−0·06
	149	+0·01	0·00

* Suspensions all of equal turbidity.

A second experiment showed that an alkaline reaction was obtained, as before, with 4 other *Pseudomonas* cultures, 2 pigmented and 2 non-pigmented, whereas 3 further strains of *Achromobacter* failed to produce any change (Table 5). No change took place when boiled *Pseudomonas* suspensions were used, and growth did not occur during the experiment. The Thunberg tubes were made to fit into an EEL nephelometer, and readings were made on a duplicate set of tubes containing no indicator: these showed that there was no increase in turbidity during the period of incubation.

These results showed that *Pseudomonas* cells, even when grown with aeration, contained enzymes which would lead to the production of an alkaline reaction in medium 1 under anaerobic conditions, without growth occurring, whereas the *Achromobacter* cells did not. The difference therefore lay in the enzymic constitution of the bacteria and not merely in their ability to grow at reduced oxygen tensions.

Experiments with washed suspensions and amino acids

An attempt was made to identify the ingredient in the Difco peptone which gave rise to the alkaline reaction, and initially 24 amino acids in groups (Table 2) were used as substrates. As well as 2 pigmented (nos. 11 and 145A) and 3 non-pigmented (nos. 155A, 141 and 167) *Pseudomonas* cultures from chicken meat, 1 pigmented culture from water (VGC/1) and 1 from soil (D/418) were tested. So also was a culture of *Alcaligenes bookeri* (NCIB 8155), another organism which had been found to give alkaline conditions in the peptone medium. The incubation periods varied between 18 and 20 hr, and slight pH changes of less than 0·2 units were disregarded. To summarize the results, all the cultures produced alkaline conditions only with medium 1 and arginine as substrates. Of the pH changes in medium 1 the smallest was recorded for *Alcaligenes bookeri* (+0·26 units), while the *Pseudomonas* strains, in suspensions of the same turbidity, produced changes from +0·55 to +0·74 units. With arginine as substrate, no buffer being present, the amount of change was +1·3 units with *Alcaligenes bookeri*, and with the *Pseudomonas* strains it varied from +1·15 to +1·65 units.

It was thought that arginine might be decomposed by the arginine dihydrolase enzyme system (see Table 8 and Discussion). It was shown by Knivett (1952*a*) that with *Streptococcus faecalis* cells treated with cetyltrimethylammonium bromide (CTAB), the reaction proceeded only as far as the intermediate product, citrulline. This could be estimated by the colorimetric method of Archibald (1944), used in a modified form by Knivett (1952*b*). Accordingly, CTAB treated suspensions of the pigmented *Pseudomonas* strain 145A and the non-pigmented strains 155A, 141 and 167 were incubated with an arginine-buffer mixture, as described by Knivett (1952*b*). After 1 hr at 25° the colour test showed the presence of citrulline in the mixture with CTAB treated cells, and its absence in that with untreated cells. This effect was the same in tubes incubated aerobically and anaerobically. It therefore appeared that the arginine dihydrolase enzymes were present in the pigmented and the non-pigmented organisms tested.

Experiments with arginine media

An arginine containing medium was required, to provide a simple test for large numbers of cultures. Medium 2 (Table 1) was derived from medium 1 by reducing the peptone concentration to the very low value of 0·1%, and medium 2A was derived from medium 2 by adding 1% of arginine. The reduction in peptone concentration was designed to minimize any interference with reactions due to breakdown of arginine.

In spite of the low concentration of nutrients in medium 2, almost all the strains inoculated produced visible growth in it.

Open and closed bottles of media 2 and 2A were inoculated with a variety of organisms, and some of the results obtained are shown in Table 6. In medium 2 all the *Pseudomonas* cultures formed alkali in the open bottles, with no change or a very slight alkaline shift in the closed bottles. Its buffer action was presumably sufficient to cope with any alkali produced from the small amount of peptone present. In the arginine medium a strongly alkaline reaction appeared in both the open and sealed bottles after 2–3 days incubation. One strain of *Ps. formicans* and 3 of *Aeromonas* gave the same reaction. With 10 of the *Pseudomonas* cultures from chicken, and with *Ps. formicans* and the *Aeromonas* cultures, tests were done with Nessler's reagent on the closed bottles of media 2 and 2A. These showed that ammonia was present after incubation only in the arginine containing medium.

Table 6. *A comparison of the reactions produced by various Gram-negative organisms in medium* 2 (*without arginine*) *and medium* 2A (*with added arginine*)

Organism	Source or collection no.*	No. of cultures tested	Reactions† in medium‡			
			2, with bottle		2A, with bottle	
			Open	Closed	Open	Closed
Pseudomonas (pigmented)	Chicken	8	Alk. or slight alk.	Slight alk. or NC	Alk.+	Alk.+
Pseudomonas (non-pigmented)	Chicken	18				
Ps. formicans	Shewan	1				
Aeromonas hydrophila	NCTC 7810	1				
Aero. hydrophila	NCTC 7812	1				
Aero. liquefaciens	L417 (Shewan)	1				
Achromobacter	Chicken	27	Alk. or slight alk.	NC or acid	Alk. or slight alk.	NC
Alcaligenes viscosus	NCIB 8154	1				
Alc. viscosus	NCIB 8596	1				
Alc. faecalis	NCIB 8156	1				
Alc. faecalis	NCTC 415	1				
Flavobacterium aurantiacum	NCIB 8204	1				
Flav. denitrificans	NCIB 8205	1				
Achromobacter lacticum	NCIB 8208	1	Alk.	NC or acid	Alk.+	NC or acid
Flav. flavescens	NCIB 8187	1				
Flav. esteroaromaticum	NCIB 8186	1				
Flav. aquatile	NCIB 8748	1				
Gas producing fermenters	Chicken	4	Alk.	NC or acid	Alk.	NC or acid

* NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures. † Alk., alkaline reaction; NC, growth with no change in reaction; Alk.+, a stronger reaction with arginine present than in the corresponding bottle without arginine. ‡ Medium 2 had only 0·1% of peptone; medium 2A contained 1% of arginine in addition.

In medium 2, as would be expected, the *Achromobacter* cultures tested gave alkaline shifts, often only slight, in the open bottles, and no change or slight acidity in the closed bottles (Table 6). In many cases, the results in the arginine medium were exactly the same. It would seem that these strains did not utilize arginine, or not in such a way as to cause a marked pH change. As well as *Achromobacter*, strains of some species of *Alcaligenes* and *Flavobacterium* fell into this group. A slight variation of this behaviour occurred with *Achromobacter lacticum* and several other *Flavobacterium* species. Here, the open bottles of the arginine containing medium 2A developed a strongly alkaline reaction, much in excess of that in the open bottles without arginine, and ammonia was present in the arginine medium. No alkaline shift took place in the closed bottles, however.

A few gas producing fermenters of the coli-aerogenes group were also tested; they all produced a strongly alkaline reaction under aerobic conditions, and no change or acid conditions in the sealed bottles.

It seemed of little interest, from these results, to continue to compare the results in open and closed bottles of media with and without arginine, as the aerobic conditions of the open bottles allowed the possibility of too many alkaline reactions. In the closed bottles, an alkaline reaction from the peptone alone had not been observed without a corresponding arginine reaction; therefore, for screening a large number of cultures, an alkaline pH shift in the sealed bottle of the arginine medium 2A was considered sufficient evidence of arginine breakdown of the type brought about by *Pseudomonas* strains.

Tests on Gram-negative bacteria from other sources in arginine, peptone and glucose media

Tests in the arginine medium, the peptone containing medium 1 (closed bottle) and the Hugh & Leifson glucose medium were applied to a large number of Gram-negative bacteria from culture collections, with particular attention to strains of *Pseudomonas* and *Achromobacter*. These results are summarized in Table 7, where the organisms are grouped according to their reactions in the arginine medium 2A in closed bottles, group I forming alkali and group II giving no change or slight acidity. It can be seen that the production of alkali separates most *Pseudomonas* species and strains from nearly all the rest. An exception was *Alcaligenes bookeri* NCIB 8155, which gave an alkaline reaction after 14 days. However, most *Pseudomonas* strains gave that reaction in 3 days or less. Also, this organism could easily be distinguished from a *Pseudomonas* by its failure to oxidize glucose. Three glucose-fermenting *Vibrio* species also fell into group I, while other *Vibrio* species occurred in group II (section B). Two halophilic *Vibrio* strains, G220 and RS368, supplied by Dr. Shewan and 5 *Vibrio paracholerae* strains (NCTC 30, 4711, 4715, 4716 and 8042) also fell in group II but their behaviour in the other conditions was not tested. *Chromobacterium violaceum* NCTC 9757 also produced the alkaline reaction, but differed from *Pseudomonas* in pigmentation and in fermenting glucose. A number of other *Chromobacterium violaceum* strains which oxidized glucose failed to give the alkaline reaction (group II, section A, Table 7). One strain of *Ps. formicans* and 10 *Aeromonas* cultures gave strongly alkaline reactions in the arginine medium, but slight acid and no change respectively in the peptone medium 1 in closed bottles. As the results obtained with 2 strains of *Cloaca cloacae* were the same as with the *Aeromonas* cultures this test is evidently of no use for distinguishing the fermentative polarly flagellated group from members of the Enterobacteriaceae.

Table 7. *The association between the reactions produced in closed bottles of medium* 2A *by Gram-negative bacteria from culture collections, and those produced in Hugh & Leifson's glucose medium and in closed bottles of medium* 1

Organism	No. of cultures tested	Designation* and source	Reaction produced**		
			Glucose, open	Glucose, closed	Medium 1 closed
Group I. Organisms producing alkali in closed bottles of medium 2A.					
Pseudomonas aeruginosa	3	NCTC 6750, 8060, 5083	Acid	Alk.	Alk.
Ps. fluorescens	4	NCIB 3756, 8251, 8248, NCTC 4755			
Ps. ovalis	1	NCIB 8296			
Ps. fragi	1	NCIB 8542			
Pseudomonas strains, pigmented	9	Human plasma (Rhodes)			
	10	Sewage (Rhodes)			
	10	Soil (Rhodes)			
	10	Milk (Rhodes)			
	1	Fish, 1005 (Shewan)			
	1	Soil, C 123B (Paton)			
	1	P6009–2 (Brenner)			
	10	Lake water (Collins)	Acid	Acid‡	Alk.
Pseudomonas sp.	1	NCTC 7452	Alk.	Alk.	Alk.‡
†*Alcaligenes bookeri*	1	NCIB 8155	Alk.	Sl. alk.	Alk.‡
Vibrio ichthyodermis	1	SH1 (Shewan)	Acid	Acid	No change
V. anguillarum	1	(Shewan)	Acid	Acid	No change
Vibrio sp. Smith & Krueger	1	(Shewan)	Acid	Acid	Alk.
Chromobacterium violaceum	1	NCTC 9757	Acid	Acid	Alk.
Pseudomonas formicans	1	(Shewan)	Acid	Acid	Sl. acid
Aeromonas hydrophila	2	NCTC 7810, 7812	Acid and gas	Acid and gas	No change
Aero. liquefaciens	1	L417 (Shewan)			
Aeromonas strains	7	(Barnes)			
Cloaca cloacae	2	NCTC 8155, 8168			
Group II. Organisms producing no change or acid in closed bottles of medium 2A.					
A. Organisms which oxidize glucose to acid.					
Pseudomonas mors-prunorum	1	330 (Dowson)	Acid	No change	Sl. acid
Ps. phaseolicola	1	52 (Dowson)			Sl. acid
Achromobacter anitratus	1	NCTC 8102			No change
Achr. lacticum	1	NCIB 8208			No change
Achr. hartlebii	1	NCIB 8129			No change
Achromobacter strains	17	Lake water (Collins)			No change
Flavobacterium suavolens	1	NCIB 8188			No change
Flav. flavescens	1	NCIB 8187			No change
§*Flav. esteroaromaticum*	1	NCIB 8186			No change
Flav. denitrificans	1	NCIB 8205			Sl. acid
Flav. aquatile	1	NCIB 8748			Sl. acid
§*Flavobacterium* strain	1	F31 (Windle Taylor, Shewan)			No change
Chromobacterium violaceum	5	Lake water (Collins)			No change
B. Organisms which do not form acid from glucose.					
Pseudomonas diminuta	1	NCTC 8545	Alk.	No change	No change
Pseudomonas (non-pigmented)	1	8/6 (Rhodes)			Sl. acid
Achromobacter strains	2	A 1011, NC 80 (Shewan)			Sl. acid
Alcaligenes viscosus	2	NCIB 8154, 8596			No change
Alc. faecalis	2	NCIB 8156, NCTC 415			No change
Alc. denitrificans	1	NCTC 8582			No change
Alc. tolerans	1	NCIB 8552			Acid
Flavobacterium aurantiacum	1	NCIB 8204			No change
§*Flav. sulphureum*	1	NCIB 8207			Acid
‖*Flavobacterium* strains	3	L 624, MacLeod A 18, G 1566 (Shewan)			Acid
Vibrio cyclosites	1	NCIB 2581			Sl. acid
V. neocistes	1	NCIB 2582			Sl. acid
V. percolans	1	NCIB 8193			Sl. acid
§*V. alcaligenes*	1	NCTC 7048			Acid
C. Organisms which ferment glucose.					
Flavobacterium proteus	1	NCIB 8771	Acid	Acid	Acid
Chromobacterium prodigiosum	1	NCTC 1377	Acid	Acid	
Escherichia coli	3	B and B/r (Alper); C_3, NCTC 8193	Acid and gas	Acid and gas	
Proteus vulgaris	1	LTRS collection			
Aerobacter aerogenes	2	NCIB 8017, 8521			
Chromobacterium prodigiosum	1	NCTC 8706			

* NCTC, NCIB, National Collections of Type Cultures and Industrial Bacteria respectively; LTRS, Low Temperature Research Station, Cambridge. Other designations are those of the workers listed, who supplied them.

** Alk., alkaline; Sl., slight. ‡ These reactions developed slowly.

† Formed alkali slowly and to a relatively small extent in medium 2A.

§ These organisms produced only slight acidity in closed bottles of Hugh & Leifson's glucose medium.

‖ These strains produced no change in open bottles of Hugh & Leifson's glucose medium.

The cultures which did not give rise to alkali in medium 2A (group II) were further subdivided, for convenience, according to their action on glucose. Those which oxidized it to acid were placed in group IIA, those which failed to give acid from it in group IIB and those which fermented it in group IIC.

Two *Pseudomonas* species were unusual in not giving alkali from arginine, though they produced a fluorescent pigment and oxidized glucose. These were the plant pathogens *Ps. mors-prunorum* and *Ps. phaseolicola*, which fell into group IIA. Two other *Pseudomonas* cultures, *Ps. diminuta* NCTC 8545 and *Ps.* 8/6, which produced no fluorescent pigment also gave no alkaline reaction in the arginine medium. They did not attack glucose and were placed in group IIB. As the genus *Pseudomonas* is now defined in Bergey's Manual (7th ed., Breed, Murray & Smith, 1957), only organisms which attack glucose can be included, and therefore these must be regarded as belonging elsewhere. This has already been suggested by Rhodes (1958) for *Ps. diminuta*, which she considers might be more closely related to *Vibrio* or *Acetobacter*.

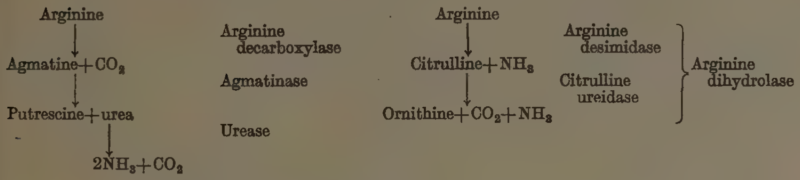
Other organisms which gave either no change or a very slight acid reaction in medium 2A and attacked glucose by an oxidative mechanism (group IIA) included many strains and species of *Achromobacter* and some of *Flavobacterium* and *Chromobacterium violaceum*. Organisms placed in group IIB, inert with glucose, included many cultures of *Achromobacter* from chicken meat (these are not shown in Table 7) and some species and strains of *Alcaligenes* and *Flavobacterium*, and also 4 *Vibrio* species. Only a few fermentative organisms (group IIC) were tested. All except the *Cloaca cloacae* strains already mentioned and placed in group I gave no change or acid in the arginine medium 2A. To sum up the results in Table 7, the *Pseudomonas* strains and species which oxidized glucose, whether pigmented or not, usually also showed the alkaline reaction in the arginine medium in closed bottles. Exceptions so far discovered are 1 pigmented culture from chicken meat, and 2 pigmented plant pathogenic species. Some organisms classed as *Pseudomonas*, but not able to produce acid from glucose, failed to give the alkaline reaction, as did the seven polarly flagellated unclassified organisms from chicken (Table 3, group E_2). An exception to this was *Pseudomonas* sp. NCTC 7452, which gave no acid from glucose and a slow alkaline reaction in medium 1 and the arginine medium, in closed bottles in both cases.

Discussion

Some of the theoretically possible mechanisms of anaerobic arginine metabolism are shown by Table 8. Arginine decarboxylase produces agmatine which may be broken down to putrescine and urea by agmatinase; and if urease is present, ammonia and carbon dioxide are formed. Breakdown as far as putrescine has been demonstrated in the Enterobacteriaceae by Moeller (1955), but has not been recorded in *Pseudomonas*.

The arginine dihydrolase enzyme system consists of arginine desimidase, which breaks down arginine to citrulline and ammonia, and citrulline ureidase, which gives ornithine, ammonia and CO_2 from citrulline, with the formation of a high energy phosphate bond. The overall reaction, shown in Table 8, was demonstrated in streptococci by Hills (1940) and the system has since been studied intensively in the streptococci (Knivett, 1952*a*, *b*, 1953; Oginsky & Gehrig, 1952; Korzenovsky &

Table 8. *Possible methods of breakdown of arginine* (*after* Moeller, 1955)



Overall arginine dihydrolase reaction:

$$\genfrac{}{}{0pt}{}{NH_2}{NH}\!\!>\!C.NH(CH_2)_3CH(NH_2).COOH+2H_2O\longrightarrow 2NH_3+CO_2+NH_2(CH_2)_3CH(NH_2)COOH$$

Werkman, 1952, 1953; Slade & Slamp, 1952). In streptococci, the incidence of arginine dihydrolase is used as a test for differentiating species (Niven, Smiley & Sherman, 1942). In the lactobacilli, it is usually present only in heterofermentative species (Briggs, 1953). Ammonia production from arginine by *Staphylococcus aureus* was noted by Hills (1940), who thought that the enzyme concerned was arginine dihydrolase. Soru (1958) detected urea as well as ornithine among the products of arginine metabolism, and concluded that arginase was responsible. Soru also stated that among staphylococci only the pathogenic strains contained arginase. An enzyme system attacking the guanidine group of arginine is present in *Bacillus subtilis* (Tomota, 1941), and enzymes of the arginine dihydrolase system have been shown in several species of *Clostridium* (e.g. Woods, 1936; Schmidt, Logan & Tytell, 1952). Some Gram-positive bacteria were tested in the peptone containing medium 1 (Table 9), and the development of alkalinity in the closed bottles showed a close correlation with the known occurrence of the arginine-decomposing enzymes described above. *Strep. faecalis* var. *liquefaciens* gave negative results, but tests on the arginine medium were positive for all the streptococci tested.

Among Gram-negative bacteria, the attack on the guanidine group of arginine was demonstrated as early as 1924 by Hino with *Pseudomonas aeruginosa* and *Ps. fluorescens*. The arginine desimidase was first described by Horn (1933) in *Ps. aeruginosa* (*B. pyocyaneus*). Arginine dihydrolase was shown by Roche, Lacombe & Girard (1950) to occur in *Ps. ovalis*, where ornithine was the end product, and in *Ps. eisenbergii*, which decomposed ornithine with the liberation of an extra molecule of ammonia. Cell free extracts of *Ps. aeruginosa* have been used by Oginsky (1955) to study the citrulline to ornithine reaction. Attack on arginine, which in some cases proved to be by the dihydrolase enzymes, has therefore been demonstrated in four species of *Pseudomonas*. It was also shown by Neilson & Eagles (1947) to occur in *Proteus ichthyosmius*, also known as *Ps. ichthyosmius* (Breed, Murray & Hitchens, 1948) and now regarded as *Aeromonas hydrophila* (Miles & Miles, 1951).

In the Enterobacteriaceae, Tomota (1940) demonstrated an attack on the guanidine group of arginine by *Salmonella enteritidis*, and Moeller (1955) showed that the products of arginine dihydrolase were formed by some strains of *E. coli*, *Cloaca* and *Salmonella*. It was usually absent in *Klebsiella* and the use of an arginine test for distinguishing between *Klebsiella* and *Cloaca* has been suggested (Moeller, 1955; Hormaeche & Munilla, 1957).

The present work has shown the prevalence in the genus *Pseudomonas* of an anaerobic enzyme system which attacks arginine. In the 4 cultures tested, 1 pigmented and 3 non-pigmented, the accumulation of citrulline by CTAB treated cells indicates the action of arginine dihydrolase, already known to occur in several pigmented species. It seems probable that this enzyme system is responsible for the rapid development of alkalinity in the arginine medium by members of the genus *Pseudomonas.*

Table 9. *The association between the reactions produced in medium 1 by Gram-positive bacteria from culture collections and those produced in Hugh & Leifson's glucose medium*

Organism	No. of cultures tested	Designation* and source	Reaction produced†			
			Medium 1		Glucose	
			Open	Closed	Open	Closed
Staphylococcus aureus	2	NCTC 7447, 8532	Alk.	Alk.	Acid	Acid
Staph. saprophyticus	1	NCTC 7292		Acid	Acid	Acid
Staph. lactis	1	NCTC 7564		Sl. acid	Acid	Acid
Staph. roseus	1	NCTC 7520		Sl. acid	NC	NC
Staph. afermentans	1	NCTC 2665		Sl. acid	NC	NC
Streptococcus faecium	2	S748, R/298 (Barnes)	Alk.	Alk.	Acid	Acid
Strep. faecalis	1	N/83 (Barnes)		Sl. alk.		
var. *zymogenes*	1	C1 (Barnes)		Sl. alk.		
var. *liquefaciens*	1	E/D76 (Barnes)		NC		
Strep. durans	2	C3, H2 (Barnes)		Alk.		
Streptococcus sp.	1	R/314, group D unclassified type 1 (Barnes)		Alk.		
Lactobacillus brevis	1	NCDO 473	Alk.	Alk.	Acid	Acid
‡*L. buchneri*	1	NCDO 110	Alk.	Alk.		
L. plantarum	1	NCDO 83	Acid	Acid		
Microbacterium thermosphactum	2	CR12A, WR7 (Niven)	Acid	Acid	Acid	Acid
Corynebacterium strains	4	EB/F/37/5, 31, 170 (Barnes)	Alk.	Acid	Acid	NC
		EB/F/37/30 (Barnes)	Alk.	Sl. acid	Alk.	NC
Bacterium linens	1	739 (Garvie)	Alk.	NC	Alk.	NC
Kurthia zopfii	2	NCTC 404, 405	Alk.	NC	Alk.	NC
Bacillus subtilis	2	LTRS collection	Alk.	Sl. acid	Acid	Sl. acid

* NCTC, NCDO, National Collections of Type Cultures and Dairy Organisms respectively; LTRS, Low Temperature Research Station, Cambridge. Other designations are those of the workers listed, who supplied them.

† Alk., alkaline reaction; Sl., slight; NC, growth with no change in reaction.

‡ Not tested in open bottles of Hugh & Leifson's glucose medium.

The anaerobic production of alkalinity from arginine was also effected by the 10 strains of *Aeromonas* which were tested, and by several *Vibrio* species, a mesophilic strain of *Chromobacterium violaceum* and one species of *Alcaligenes*, where it has not hitherto been described.

Of the various methods used in this work, the test recommended for screening a large number of cultures employs the arginine medium 2A (Table 1), autoclaved in approximately 3 ml amounts in 5 ml bottles. Each organism is inoculated by stabbing into one bottle, which is then sealed with sterile melted vaseline and incubated. Colour changes of the indicator in inoculated bottles, compared with a control, are noted, and a pH change towards the alkaline side is regarded as a positive result. Nearly all *Pseudomonas* strains gave a positive result after 3 days at 20°, and the slowest alkaline change (for *Alcaligenes bookeri*) was recorded after 14 days.

The incubation temperature of 20° was chosen for the psychrophilic organisms studied here, but this could be varied according to the nature of the organisms.

It is suggested that this test may prove useful for distinguishing *Pseudomonas* from other Gram-negative glucose-oxidizing bacteria, particularly the *Achromobacter* group. The occurrence of the same reaction in several groups with a different type of glucose metabolism makes it imperative to combine this test with Hugh & Leifson's test; and since exceptions to the arginine test have already been shown to occur in undoubted *Pseudomonas* species, it remains desirable to use these two tests in conjunction with as many others as possible.

This work has been carried out as part of the programme of the Low Temperature Research Station, Cambridge.

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FACTORS CONTROLLING THE SPORULATION OF YEASTS. I. THE PRESPORULATION PHASE

By R. R. FOWELL and MARGARET E. MOORSE

The Distillers Company Limited, Research and Development Department,
Great Burgh, Epsom, Surrey

SUMMARY: Yeasts tend to dissociate into mixtures of cell types with different powers of sporulation; hence single cell isolates are recommended for sporulation studies. The ability of yeasts to produce 4-spored asci can be improved by single cell selection. Cells from actively fermenting cultures sporulate much better than those grown under aerobic conditions. Sporulating ability depends on fermentation 'age', reaching a maximum when 85–90% of the CO_2 has been evolved. Carbon dioxide assimilation in the presporulation phase appears essential for maximal sporulation, but complete anaerobiosis in this phase is detrimental to sporulating ability. Malt wort cultures of a baker's yeast have given remarkably constant figures, in successive tests, for sporulation; but some batches of wort have an adverse effect on sporulating ability. The same yeast, grown on Lodder-Rij's synthetic medium containing 4 or 8% (w/v) of glucose, is capable of 80% sporulation (proportion of cells forming asci) on sodium acetate agar, comparable to that obtainable with malt wort cultures. Sporulation is depressed by excess storage of fat, while storage of glycogen does not affect sporulating ability.

The sporulation of yeasts has been the subject of considerable research since the classical review of Phaff & Mrak (1948, 1949), and Ingram (1955) and Morris (1958) have provided useful summaries of recent work. The present paper, and the one to follow, represent an attempt to appraise, in the light of our own researches, the conditions that govern sporulation.

It is important to distinguish clearly, in experimental studies, between the presporulation and sporulation phases, which are governed by entirely different conditions; distinct separation of the two phases is necessary, not only for rapid induction of spore formation, but also for valid assessment of the factors that operate in the two phases. The presporulation phase is essentially an anaerobic growth stage; the sporulation phase is aerobic and characterized by restricted cell division.

The factors that control sporulation may be classified as genetical, chemical and physical. Yeasts vary enormously in their powers of sporulation, and the sporulating ability of many yeasts is markedly influenced by environmental conditions. However, under controlled conditions, including the use of a standard sporulation medium, the degree of sporulation is highly characteristic for each strain of yeast. The spore diagram, which refers to the proportions of asci with different numbers of spores, is an even more reliable feature for taxonomic purposes (Oppenoorth, 1957), as we can confirm; variations in conditions may affect the degree of sporulation without much disturbance to the form of the spore diagram. On different sporulation media, however, the same yeast may show quite different spore diagrams (Fowell, 1955; Oppenoorth, 1957). These observations indicate that, though sporulation is genetically controlled, the degree of sporulation as well as the spore diagram are considerably influenced by environmental conditions.

Any study of the effect of such conditions on sporulation should ideally be based on yeasts with a fixed genetical constitution. Many yeasts tend to lose their sporulating ability, especially when maintained on artificial media (Lindner, 1896). Decline in sporulating ability appears to be due to mutation, and to the more vigorous growth exhibited by sporeless mutants (Hansen, 1900; Olenov, 1936). Leupold (1950) found that a culture of *Schizosaccharomyces pombe*, after repeated transfer, was a mixture of three cell types distinguished by different powers of sporulation as 0, 40, and 90% types. These observations indicate that yeasts maintained for long periods are liable to become mixtures of sporulating cell types.

Studies of presporulation conditions have been mainly concerned with chemical factors. As first mentioned by Hansen (1883), and confirmed by later workers, maximal sporulation depends on the use of healthy, well nourished cells for the inoculation of the sporulation media. Special presporulation media, commonly mixtures of fruit and vegetable juices, have been recommended for the invigoration of yeasts prior to sporulation (e.g. Baltatu, 1939; Mrak, Phaff & Douglas, 1942; Lindegren & Lindegren, 1944); but such recommendations have not been supported by adequate experimental data.

Wagner (1928) reported that the degree of sporulation is independent of the sugar concentration in the presporulation medium over a wide range. This finding, however, is open to doubt because his sugar solutions were supplemented by 10% of yeast extract, which contains sufficient trehalose to support good growth of yeasts (Myrbäck & Örtenblad, 1936); furthermore, he used unwashed cells to inoculate sporulation media, and the resultant transfer of variable amounts of nutrients and waste products from the growth media may have had considerable influence on sporulation.

Ochmann (1932) tested the effect of the presence of various nitrogen compounds during growth on subsequent sporulation, and found that some were far more favourable than others. Thus a strain of *Saccharomyces cerevisiae*, following growth in a basal medium containing glutamic acid, sporulated better than after growth in hopped wort which, compared with yeast protein, is deficient in this amino acid (Block & Bolling, 1945; Barton-Wright & Thorne, 1949). But caution is needed in assessing his findings, as he used unwashed cells to inoculate the sporulation media, and his yeasts were grown in a basal medium lacking in vitamins which markedly influence the utilization of nitrogen compounds (Phaff & Mrak, 1949). Tremaine & Miller (1956) rectified these deficiencies of technique, and their results showed that in general nitrogen compounds capable of supporting good growth are the most effective for subsequent sporulation.

Stantial (1935) first demonstrated the importance of vitamins for sporulation. Subsequent workers found that yeast extract or autolysate, rich in B vitamins, promoted sporulation. Tremaine & Miller (1954) found that maximal sporulation, and the production of the highest number of 4-spored asci, depended on the presence of a full complement of B vitamins in the presporulation medium; under these conditions, no advantage accrued from the addition of vitamins to the sporulation medium, with the exception of pantothenate which, it was claimed, gave increased yields of spores.

Thermolabile substances such as enzymes are destroyed when growth media are heat sterilized. Tsukahara & Yamada (1953) claimed that sporulating ability in saké yeasts can be restored by serial transfer in raw koji extract sterilized by filtration instead of heat. According to these authors, enzymes and other thermolabile substances in the koji extract were necessary for sporulation.

Little attention has been devoted to the effect on sporulation of the physical conditions during presporulation growth. Tremaine & Miller (1954) found that liquid grown cells sporulated much better than those grown on solid media, but Kleyn (1954) reported the contrary. Aeration of presporulation cultures is detrimental to sporulation (Pazonyi, 1954; Fowell, 1955), which indicates that yeasts grown under anaerobic conditions show the highest degree of sporulation.

The effect of presporulation temperature has not, in our opinion, been properly investigated. Growth at a high temperature, near the maximum for budding, is detrimental to sporulation (Hansen, 1900; Adams & Miller, 1954), though according to Tsukahara & Yamada (1953) saké yeasts must be grown near the maximum temperature in order to restore sporulating ability. Adams & Miller (1954) incubated two yeasts at different temperatures, but for a constant period of 48 hr, and claimed that the most suitable temperature for subsequent sporulation was 30° or less. However, they failed to take account of the effect of temperature on the physiological age of yeast cells, which influences sporulating ability: it is well known that cells from young cultures give the highest yield of spores (Hansen, 1883; Barker, 1902; Miller, Calvin & Tremaine, 1955). Miller *et al.* (1955) have shown that the decline in sporulating ability of old cultures is due, not to an increase in the number of dead cells, but to physiological changes in the cells.

A different approach to the study of the effect of presporulation treatment is to consider the influence on sporulation of variations in the chemical composition of yeast cells. Chemical composition is determined by physical as well as chemical conditions of growth. Thus under aerobic conditions protein content increases and carbohydrates decrease, while in anaerobic cultures these changes are reversed. During fermentation glycogen accumulates in yeast cells, and this has led to the general belief that ample reserves of glycogen are necessary for sporulation. However, Pazonyi & Markus (1955), using the sensitive anthrone reagent for carbohydrate estimation, found that a sporulating culture actually had a higher carbohydrate content than a vegetative culture, while the glycogen content remained unchanged after sporulation. The increase in carbohydrate is mainly due to larger amounts of mannan and glucan, which is accounted for by the increase in the cell wall fraction consequent upon spore formation. The above authors concluded that glycogen does not play the important part in sporulation previously attributed to it. It has recently been stated (Schanderl, 1957) that storage of fat is essential for sporulation, but no experimental data have been supplied in support of this claim.

MATERIALS AND METHODS

Yeasts. Three single cell isolates of a baker's yeast, DCL 379, 568 and 740, were used in most of the experiments. Other yeasts used were a brewer's yeast, DCL 552; a wine yeast, DCL 222; and another baker's yeast, DCL 369. All the above yeasts were strains of *Saccharomyces cerevisiae*. They were maintained at 30° on malt wort, containing 16% (w/v) of malt extract.

Sporulation technique. Standardization of this technique was necessary for the proper assessment of the effects of variations in presporulation treatment.

The sporulation medium used contained 0·5% (w/v) of anhydrous sodium acetate and 1·5% (w/v) of agar (Fowell, 1952); after autoclaving, this medium has a pH of 6·8±0·2. Yeast cells were washed twice with distilled water before transfer to sodium acetate agar slopes. The inocula were adjusted to give a cell density of $0·5–1·0 \times 10^6$ cells/ml of acetate agar. Slopes, after inoculation, were incubated for 5 days at 25°. Figures for sporulation were based on counts of 500 cells, but where the degree of sporulation was low up to 1000 cells were counted.

Presporulation media. One litre aliquots of malt wort, containing 16% (w/v) of malt extract, were used as standard growth media, each aliquot being inoculated with 10 ml of a culture grown on the same medium and then incubated at 30°. Lodder-Rij's liquid synthetic medium for carbon assimilation tests (Lodder & Kreger-van Rij, 1952) was adapted for use as a presporulation medium by the incorporation of 8% instead of 0·5% (w/v) of glucose; 100 ml aliquots of this medium were inoculated with twice washed cells derived from 1 ml samples of malt wort cultures. Other levels of glucose, however, were used to test the effect of sugar concentration on subsequent sporulation.

The tomato juice broth tested as a presporulation medium had the following composition: filtered tomato juice, 150 ml; yeast extract, 6 g; peptone, 15 g; NaCl, 3 g; glucose, 20 g; and distilled water to 1 litre. Solid growth media were prepared by the addition of 1·5 or 2·5% (w/v) of agar to malt wort and tomato juice broth.

A nitrogen-free glucose succinate-buffered medium (Trevelyan & Harrison, 1956) was used in a study of the influence of glycogen on sporulation, to promote glycogen storage.

Lundin's (1950) two stage cultivation technique was used, with certain modifications, to promote fat storage in the baker's yeast DCL 369, in an attempt to confirm the theory (Schanderl, 1957) that fat storage is essential for sporulation. In the first (growth) stage, 120 ml of malt wort, after inoculation, were aerated for 10 hr at 30°. The air flow in this, and in the second stage, was adjusted to 500 ml/100 ml of medium/min (Steinberg & Ordal, 1954). Washed cells from the wort medium were then transferred to 10% (w/v) sucrose plus 0·1% (w/v) KH_2PO_4 (basal medium), basal medium plus 1% (w/v) of sodium acetate, and basal medium plus 0·6% (w/v) of ascorbic acid (Maguigan & Walker, 1940). Flasks containing these media (lacking nitrogen) were aerated for 43 hr at 30°.

Sintered gas bubblers (porosity 1) were used for passing humidified air and other gas through media; the rate of flow was controlled by means of a rotameter and gas valve.

Carbohydrate estimations. Changes in the carbohydrate composition of yeasts during the fermentation of malt wort were estimated with the anthrone reagent by the method of Trevelyan & Harrison (1952).

RESULTS

Genetical factors

In 1955 a 48 hr malt wort culture of the brewery yeast DCL 552 gave 65·8% sporulation (percentage of cells with spores) on sodium acetate agar, but only 9·4%

of the cells produced 4-spored asci (Fig. 1). Its spore diagram showed a predominance of 2-spored asci. Two years later, this yeast gave only 9·2% sporulation, 4-spored asci forming 4·6% of the cell population, and the decline in sporulating ability was due mainly to a reduction in the numbers of 1- and 2-spored asci. Single cells were isolated from DCL 552 by micromanipulation and eighteen isolates were tested for sporulating ability: percentage sporulation ranged from nil to 32·7. These results indicated that the brewery yeast was a mixture of sporulating cell types. Examination of the spore diagrams revealed that the single cell isolates could be grouped into four fairly well defined cell types (Fig. 1). The spore diagrams of these were distinguished by asci containing 4, 3 and 2 spores respectively in the largest numbers; the fourth cell type was a sporeless mutant. Isolates of the first type gave the best figures for sporulation, but the highest figure recorded, 32·7%, was only half that achieved by the parent yeast two years previously; however, at least four isolates gave larger numbers of 4-spored asci, namely 13–16% of the cell population compared with 9·4% for the parent yeast in 1955.

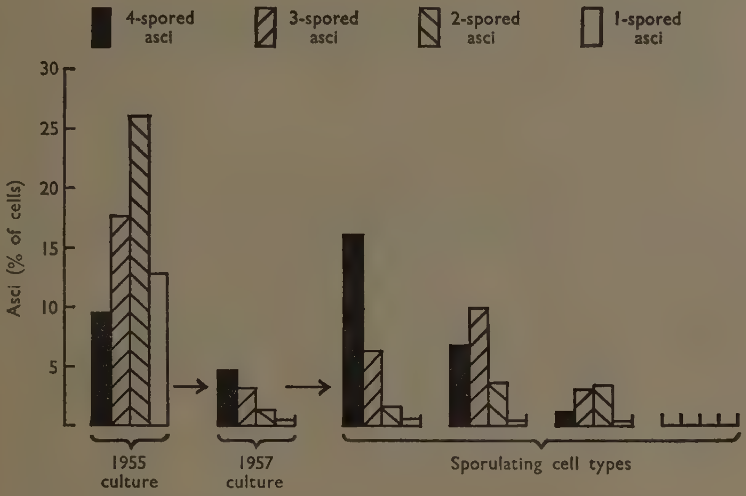

Fig. 1. Dissociation of the brewery yeast DCL 552 into a mixture of sporulating cell types distinguished by different spore diagrams. These cell types are classified into four groups, one of which embraces sporeless mutants.

In a further experiment two of the above isolates, which gave 24·0 and 32·7% sporulation respectively on sodium acetate agar, were tested on another sporulation medium, Difco nutrient agar plus NaCl (0·8 g/l.), pH 7·6. Contrary to expectation, the isolates proved inferior to the parent yeast on this medium, giving 3·5 and 5·0% sporulation respectively compared with 16·6% for the parent yeast. These results suggest that sporulating cell types may differ in their reaction on different sporulation media.

Evidence for the existence of different sporulating cell types has been found in four other yeasts. Several sporulation tests, carried out on a selected single cell isolate of DCL 369, have confirmed that this isolate is capable of producing 6–7% of 4-spored asci on sodium acetate agar compared with 2–3% for DCL 369, although there is no difference in overall sporulation. It is evident, therefore, that the ability of a yeast to produce 4-spored asci, so essential for genetical analysis, can be improved by single cell selection.

The demonstrated tendency of yeasts to dissociate into mixtures of sporulating cell types led us to decide on the use of single cell isolates in subsequent sporulation studies; the effects of environmental conditions were therefore assessed against a reasonably fixed genetical background.

Physical factors

Liquid versus solid presporulation media. Owing to conflicting reports in the literature about the relative merits of liquid and solid growth media, as they affect sporulation, experiments were carried out with DCL 568 grown on malt wort and tomato juice broth, and on corresponding agar media. Sporulation counts (Table 1) confirmed that liquid media are definitely superior, in agreement with Tremaine & Miller (1954). In spite of the use of malt agar of varied composition, and different periods of incubation, the highest figure for sporulation following growth on this medium was only 27·5%, compared with 68·3% for a control wort culture.

Table 1. *A comparison of the effect of growth in liquid and on solid presporulation media on the subsequent sporulation of* DCL 568 *at* 30°

Presporulation medium*	Incubation period (hr)	Asci (% of cells)	
		Total	4-spored
Tomato juice broth	70	36·2	24·5
Tomato juice agar	70	24·5	14·3
Malt extract 16%	40	68·3	48·0
Malt extract 16%, agar 2·5%	24	27·5	19·8
Malt extract 5·5%, agar 2·5%	20	22·2	12·0
	43	14·7	11·3
Malt extract 2%, agar 5%	20	11·2	6·4
	43	12·8	7·6

* % w/v.

Fermentation age. Preliminary experiments indicated that yeasts allowed to ferment out on malt wort do not sporulate as well as actively fermenting cultures. It was therefore decided to investigate the sporulating ability of yeast cells at intervals during the course of fermentation. DCL 568, a relatively fast fermenter of malt wort, and DCL 222, a slow fermenter, were selected for study.

One litre aliquots of malt wort, after inoculation, were incubated at 30°. Twice daily the fermenting cultures were shaken by hand and 20 ml aliquots withdrawn for sporulation tests.

DCL 568 showed maximal sporulation after 40 hr fermentation, and this period of incubation was accordingly used in subsequent experiments. Sporulation declined

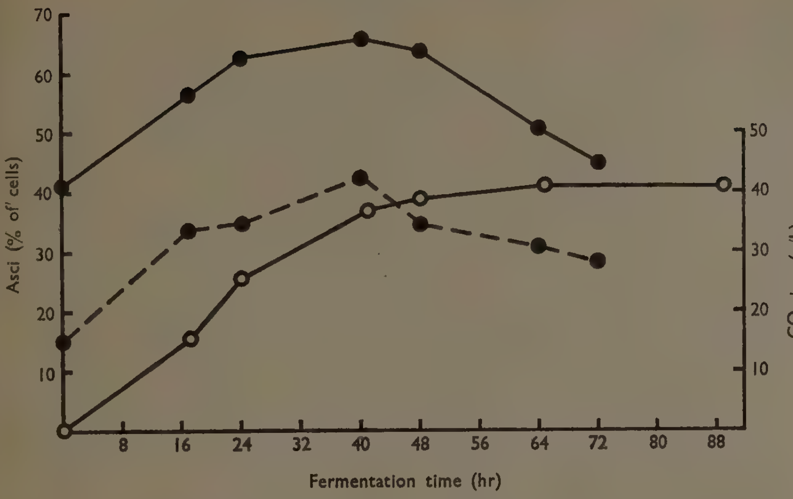

Fig. 2. The effect of fermentation age on the sporulation of a baker's yeast, DCL 568. Full circles, asci; continuous line, total asci; broken line, 4-spored asci; open circles, CO_2.

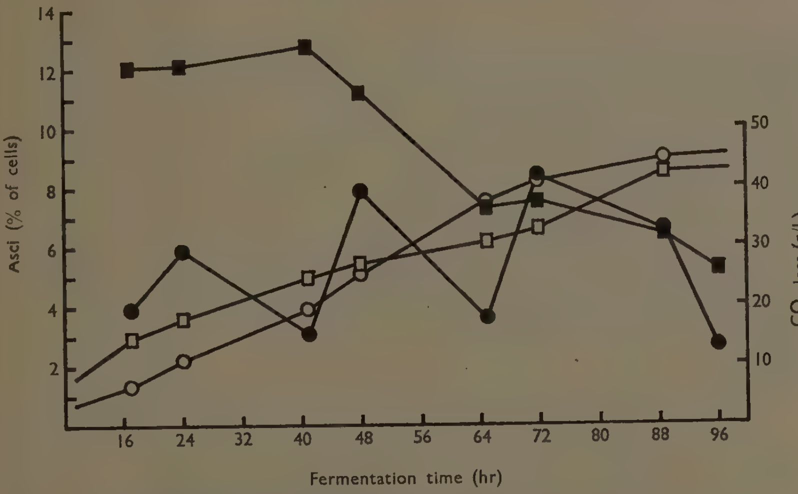

Fig. 3. The effect of fermentation age and method of agitation on the sporulation of a wine yeast, DCL 222.
Circles, hand shaken culture; full circles, asci; open circles, CO_2. Squares, magnetically stirred culture: full squares, asci; open squares, CO_2.

from a maximum of 66·1% to 50·6% at the end of fermentation, i.e. 65 hr (Fig. 2). DCL 222 sporulated best after 72 hr fermentation (Fig. 3), i.e. 32 hr later than DCL 568. However, measurements of CO_2 loss revealed that both yeasts reached the peak of sporulating ability at about the same stage in fermentation, namely when 85–90% of the CO_2 had been evolved. DCL 222 showed a marked decline in sporulating ability after 89 hr, long before fermentation ceased some 70 hr later.

DCL 222 differed from DCL 568 in exhibiting an alternate rise and fall in sporulating ability. The relatively long overnight period of 17 hr between daily shakings probably allowed the fermenting wort to become supersaturated with CO_2, with a consequent adverse effect on sporulating ability; the above alternation disappeared as soon as CO_2 evolution started to slacken. In a repeat experiment an attempt was made to prevent supersaturation of the wort with CO_2 by the use of a magnetic stirrer, and by this means the dip in the sporulation curve between recordings for 24 and 40 hr was successfully eliminated (Fig. 3); but the stirrer unfortunately failed to prevent a build-up of CO_2 in the later stages of fermentation, as is evident from the CO_2 curve. Although a higher maximum of 12·8% was recorded for sporulation, due to the use of a different batch of wort (a similar figure having been previously recorded for a hand shaken culture), sporulating ability fell off rapidly after 40 hr and only 7·5% was recorded for sporulation at 72 hr compared with 8·5% in the original experiment.

Anaerobiosis. The superiority of liquid over solid presporulation media for inducing a high degree of sporulation suggested that anaerobic metabolism might be more effective than aerobic in conditioning cells to form spores. The results recorded above with DCL 222, however, indicate that a high degree of anaerobiosis may adversely affect sporulating ability. Confirmation of this view was obtained by experiments carried out on DCL 740 (a single cell isolate of DCL 568), which was propagated on Lodder-Rij's medium containing 8% (w/v) of glucose. Carbon dioxide, oxygen and nitrogen, singly and in various combinations, were bubbled through this medium for 1 hr before inoculation, and for an ensuing period of 40 hr at 20°, at the rate of 400 ml gas/100 ml of medium/min.

The establishment of complete anaerobiosis by bubbling nitrogen through the culture proved markedly detrimental to subsequent sporulation (Table 2). Admixture of 1 part of O_2 with 19 parts of N_2 improved sporulation considerably; but the same degree of sporulation, 44%, was recorded when the two gases were mixed in approximately the same proportion (1:4) as in air.

Table 2. *The effect of the composition of the gas used to sparge a synthetic presporulation medium on the sporulation of* DCL 740

Gas mixture*			Asci (% of cells)	
N_2	CO_2	O_2	Total	4-spored
400	0	0	13·0	5·3
380	20	0	38·6	23·2
380	0	20	43·8	24·8
325†	0	75	44·0	28·6
0	400	0	53·4	29·0
0	380	20	52·6	27·4
0	275	125	66·8	38·0

* Volumes. Rate of flow, 400 ml/100 ml of medium/min.

† This mixture is equivalent to normal air.

The inclusion of a small amount of CO_2 in a nitrogen stream also improved sporulation. When CO_2 alone was bubbled through the culture a surprisingly high figure was recorded for sporulation, 52·4% compared with only 13·0% for a N_2-sparged culture. This suggests that CO_2 plays an active role in conditioning cells for sporulation. However, ordinary hand shaken cultures of DCL 740 grown on malt wort or Lodder-Rij's medium containing 4–8% (w/v) of glucose were capable of 80% sporulation (see later); hence CO_2 alone was not sufficient for the full development of sporulating ability. A trace of O_2 in a CO_2 stream failed to improve sporulation further; but when the proportion of O_2 was raised to 1:2 there was a still higher level of sporulation (Table 2).

Temperature. In order to detect a possible influence of the presporulation growth temperature on subsequent sporulation, allowance should be made for its effect on fermentation rate and therefore on fermentation age. The optimal presporulation period would depend on the temperature of incubation, and would have to be found by experiment.

We have not yet carried out a full investigation on these lines. However, in a preliminary experiment a malt wort culture of DCL 568 incubated for 72 hr at 21° gave 76·0% sporulation, compared with 74·3% for a culture grown for 43 hr at 30°. Thus the temperature of presporulation growth would not appear to have any effect *per se* on sporulation.

Chemical factors

Malt wort. The use of malt wort as a presporulation medium may be challenged on the grounds of variability of composition, due to the use of different batches of malt extract and possibly to variations in mashing and filtration. However, remarkably constant figures for sporulation have been recorded for successive single cell isolates of a baker's yeast propagated on malt wort. Thus in 8 sporulation tests on DCL 379 spread over four years the mean percentage sporulation was 67·7, with a standard deviation of only 1·0. DCL 568, derived from DCL 379, gave a mean of 67·9 with s.d. of 0·9, based on 5 tests spread over 3 months. However, towards the end of 1957, this isolate suddenly exhibited a new level of sporulation, and in 5 tests spread over 6 months a mean of 75·5 (s.d. 0·8) was recorded. Finally, DCL 740, derived from DCL 568, at first gave 70–75% sporulation, but in recent tests it has given a mean of 80·4%, which is comparable to the degree of sporulation following presporulation growth on Lodder-Rij's medium containing 4–8% (w/v) of glucose.

The sudden improvement in sporulation recorded for DCL 568 coincided with the introduction of fresh stocks of malt extract for mashing, and the mashing of malt extract within a few weeks of delivery. In previous years malt extract had usually been held in storage for at least 6 months before use. It is perhaps significant that unusually high figures for sporulation have occasionally been recorded for the earlier isolates mentioned above. These observations suggest that different mashes may yield worts of different quality, and early mashing of malt extract would appear to give worts capable of inducing the highest degree of sporulating ability. Care is obviously necessary in basing conclusions on figures for sporulation where a particular yeast has been propagated on worts from different mashes; but results based on worts from the same mash can be regarded as reliable enough in comparative studies of sporulation.

As malt wort is markedly deficient in glutamic acid compared with yeast protein, and in view of Ochmann's (1932) report of the favourable effect of this nitrogen compound on the sporulation of a strain of *Saccharomyces cerevisiae*, it was decided to test the effect on subsequent sporulation of DCL 740 of supplementing malt wort with glutamic acid. However, the addition of this compound at the equivalent of 0·3 g N/l. failed to effect any improvement in sporulation; 67·0% sporulation was recorded compared with 71·6% for a control culture.

Tomato juice broth. DCL 568 was transferred three times on this medium prior to a sporulation test. As previously mentioned, a malt wort culture of this yeast gave maximal sporulation when about 80% of the CO_2 had been evolved. For a comparable evolution on tomato juice broth, incubation for 70 hr at 30° was found necessary. The results of spore counts (Table 1) showed that for this strain of yeast, and contrary to the findings of Kleyn (1954), tomato juice broth was inferior as a presporulation medium.

Synthetic medium. Malt wort contains 8–9% (w/v) of sugars, mainly in the form of maltose. A synthetic medium, with a comparable amount of sugar, was prepared

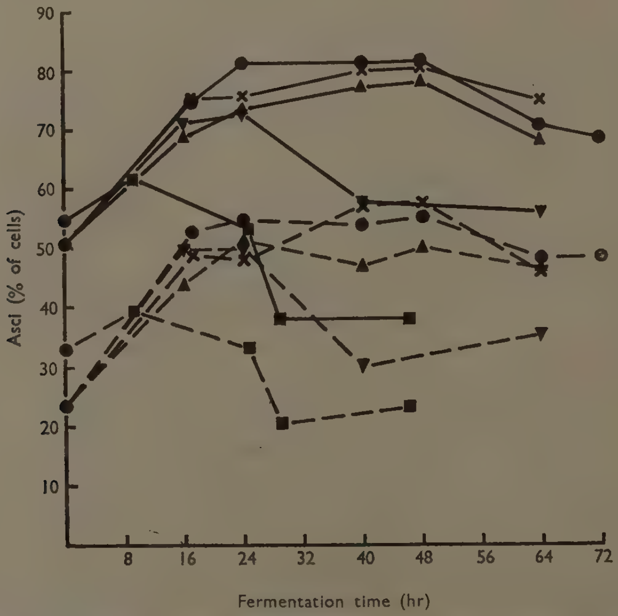

Fig. 4. The effect of sugar concentration in the presporulation medium (Lodder-Rij) on the sporulation of the baker's yeast, DCL 740. Continuous lines, total asci; broken lines, 4-spored asci.
Glucose concentration (% w/v): squares, 0·5; crosses, 4; erect triangles, 8; inverted triangles, 16. Circles, malt wort containing 8–9% (w/v) of sugar.

by adding 8% (w/v) of glucose to Lodder-Rij's medium. DCL 740, after incubation on this medium for 48 hr at 30°, gave 78·0% sporulation and 50·2% of the cells produced 4-spored asci; these figures are comparable to those obtained with recent malt wort cultures of this yeast.

Sugar concentration. DCL 740 was propagated on Lodder-Rij's medium containing respectively 0·5 (original formula), 4, 8 and 16% (w/v) of glucose. A malt wort culture was included as a control. All cultures were tested twice daily for sporulating ability, and the effect of fermentation age was again clearly demonstrated (Fig. 4). The sporulation curves for synthetic media containing respectively 4 and 8% (w/v) of glucose were essentially similar and comparable to the malt wort curve. Maximal sporulation of about 80% was recorded for presporulation periods of 40–48 hr. The culture grown in the presence of 16% (w/v) of glucose showed delayed fermentation and reduced sporulating ability; maximal sporulation of 73·0% was attained after a presporulation period of 24 hr. As expected, virtually no fermentation was observed in the presence of 0·5% (w/v) of glucose, i.e. the glucose was assimilated rather than fermented, and sporulating ability was considerably impaired; maximal sporulation of 62·0% was observed for a presporulation period of only 9 hr.

The above results indicate that the degree of sporulation is independent of the sugar concentration in the presporulation phase over a limited range only, and that extremes of sugar concentration have an adverse effect.

Effect of glycogen and fat storage

Glycogen. If, as commonly believed, glycogen plays an important part in sporulation, then variations in the glycogen content of yeast cells would be expected to influence sporulating ability.

Glycogen content, during fermentation on a nitrogen-free medium containing glucose or mannose, rises to a much higher level than in the presence of nitrogen (Trevelyan & Harrison, 1956). An attempt based on this finding was therefore made to increase the glycogen content of DCL 369 cells, in order to ascertain if the poor sporulating powers of this yeast could be improved. Ten ml of a nitrogen-free glucose medium (Trevelyan & Harrison, 1956) were inoculated with 0·5 g of wet yeast derived from a 40 hr malt wort culture. Fermentation set in rapidly, and cells were sampled at intervals for sporulation tests. The degree of sporulation fell at first but later rose slowly towards the level exhibited by the 40 hr malt wort culture. In a repeat test the nitrogen-free treatment was extended to 6·5 hr, but again the final figures for sporulation were similar to those for the control wort culture (Table 3).

Further investigations carried out on DCL 369 and DCL 740 revealed that the effect of fermentation age on sporulation was not due to variations in the carbohydrate or glycogen content of the cells. Malt wort cultures were sampled twice daily for sporulation tests and estimations of carbohydrate content, and DCL 369 showed a considerable rise in total carbohydrate content during fermentation, this being largely due to an increase in acid soluble glycogen (Fig. 5). Accumulation of glycogen coincided with a rise in sporulating ability. After 40 hr fermentation, however, while acid soluble glycogen content declined sharply sporulating ability

Table 3. *The effect of incubation in a nitrogen-free glucose medium on the sporulation of* DCL 369 *at* 30°

Incubation period (hr)	Asci (% of cells)	
	Total	4-spored
0*	7·7	2·7
2	4·6	1·8
3·3	5·7	2·3
5	7·0	3·6
6·5	7·8	2·4

* Malt wort culture incubated at 30° for 40 hr.

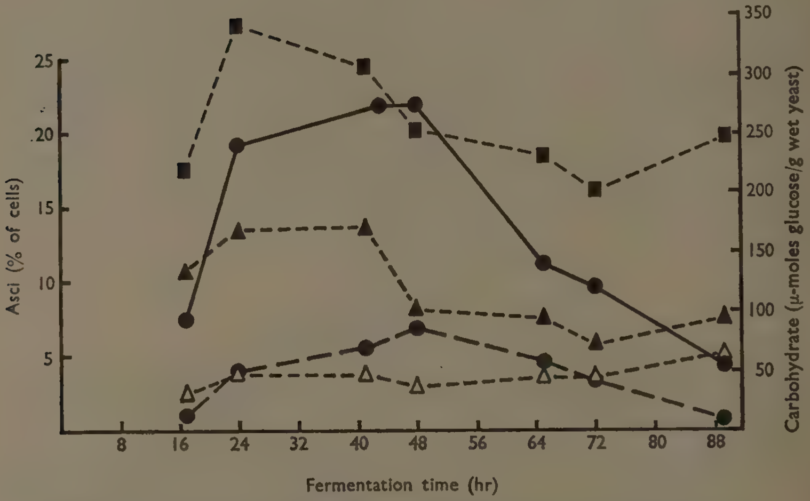

Fig. 5. The changes in the total carbohydrate and glycogen content of DCL 369 cells, and in their sporulating ability, during fermentation of a wort. Circles, asci; continuous lines, total asci; broken lines, 4-spored asci. Squares, total carbohydrate; full triangles, acid soluble glycogen; open triangles, alkali soluble glycogen.
The relatively high figures recorded here for the sporulation of DCL 369, compared with those in Tables 3 and 4, were due to the use of a large inoculum (*c.* 5×10^6 cells/ml of sodium acetate agar).

continued to improve, reaching its highest level (22%) at 48 hr; in the later stages of fermentation the acid soluble glycogen content remained virtually unchanged but sporulating ability deteriorated rapidly. DCL 740 presented a similar picture, total carbohydrate and acid soluble glycogen content showing a marked fall between 40 and 48 hr fermentation; but the degree of sporulation, 82%, recorded at 48 hr was the same as that at 40 hr. These results indicate quite clearly that maximal sporulation does not depend on glycogen storage in the presporulation phase.

Fat. Because of its poor sporulating ability, DCL 369 was used to test the theory that fat storage is essential for sporulation (Schanderl, 1957).

Table 4. *The influence of fat storage on the sporulation of* DCL 369 *at* 30°

Presporulation treatment		Asci (% of cells)	
Growth phase	Fat phase (aeration for 43 hr)	Total	4-spored
Malt wort (42 hr) (control)	—	7·5	1·7
Malt wort (10 hr) (aerated)	Cane sugar, phosphate	0·8	0·2
	Cane sugar, phosphate, acetate	0·0	0·0
	Cane sugar, phosphate, ascorbic acid	0·7	0·0

Lundin's (1950) two-stage cultivation technique was employed, with certain modifications as described above, and following this treatment the cells were found, on microscopical examination, to be packed with fat globules. Sporulation tests (Table 4) showed, quite clearly, that fat storage promoted at the expense of other types of food reserves is detrimental to sporulation.

Discussion

A tendency for yeasts to dissociate into mixtures of sporulating cell types, first observed for *Schizosaccharomyces pombe* (Leupold, 1950), has been confirmed for strains of *Saccharomyces cerevisiae*. If yeasts are maintained for long periods without selection, and such yeasts are used to study the conditions affecting sporulation, it is possible that changes in these conditions may operate by altering the proportion of cells with different powers of sporulation. It would seem advisable, therefore, to employ single cell isolates in sporulation studies.

The selection of single cell isolates, on the basis of sporulation tests, provides a rapid method of restoring sporulating ability in a yeast; it has also been found useful as a means of improving the ability of a yeast to produce 4-spored asci, so essential for genetical research. Hartelius & Ditlevsen (1956) have criticised the above method on the grounds that a single cell isolate, selected for sporulating ability, may differ in certain characteristics from the parent yeast; but this criticism could equally well apply to their technique of culture on a soil substrate, which encourages the multiplication of good sporeforming cells at the expense of poor sporeformers. If several isolates are available, selection of one or more of those with the properties of the parent yeast should present no difficulty.

In agreement with Tremaine & Miller (1954), but contrary to Kleyn (1954), it has been shown that yeasts grown in liquid media sporulate much better than those grown on solid media. Kleyn's findings are probably invalid because he failed to wash liquid grown cells prior to inoculation of sporulation media. Anaerobic metabolism, therefore, would appear to be more effective than aerobic in conditioning cells to form spores. However, complete anaerobiosis in the presporulation phase is detrimental to sporulation. This is clearly demonstrated by the effect of bubbling nitrogen through a fermenting culture of DCL 740, and the marked improvement effected in sporulation following admission of a trace of oxygen in a nitrogen stream; a similar improvement was obtained by admission of oxygen in a carbon dioxide stream. In both cases, the introduction of oxygen improved sporulating ability far beyond that of cells grown, under aerobic conditions, on solid media. The highest degree of sporulation was given by a culture sparged with a mixture of oxygen and carbon dioxide; and it was nearly as high as that given by normal malt wort cultures shaken twice daily by hand. The role of oxygen in a fermenting culture, as it affects sporulating ability, is not clear. It may be necessary for the establishment of a critical and relatively high oxidation-reduction potential. Our experiments with the wine yeast DCL 222 have revealed that the sporulating ability of this yeast is depressed by excessive amounts of carbon dioxide in the presporulation medium. Bubbling oxygen through such a medium would alleviate the inhibitory effect of carbon dioxide, not only for DCL 222 but possibly for other yeasts also. Finally, oxygen may be necessary for the promotion of growth which may, in some way, be essential for the full development of sporulating ability. None of the wort cultures of DCL 740 was subjected to gaseous treatment so that dissolved oxygen would be available to the cells only in the early stages of fermentation. The high degree of sporulation given by such cultures is probably due to persistence of the stimulatory effect of oxygen on growth, even when the cells are afterwards subjected to carbon dioxide (Brockmann & Stier, 1947).

Our experiments have shown that carbon dioxide plays an important part in preparing cells for sporulation. Thus, when carbon dioxide in place of nitrogen was bubbled through a fermenting culture of DCL 740, the subsequent degree of sporulation was quadrupled. Carbon dioxide fixation is an essential reaction for yeast metabolism as it supplies carbon for certain synthetic processes (Stoppani *et al.* 1958); it is possible, therefore, that carbon dioxide assimilation is necessary for the synthesis of materials involved in sporulation.

Special attention is drawn to the effect of fermentation age on sporulating ability. In comparative studies of sporulation it is clearly necessary to allow for differences in fermentation rates in different yeasts, and to ascertain, by experiment, the optimal incubation period for subsequent sporulation.

Although consistent figures for sporulation have been recorded for yeasts grown on malt wort, we have found evidence that certain batches of wort have a slightly adverse effect on sporulation; we have also noticed that decline in sporulating ability towards the end of fermentation is more rapid in some wort cultures. These observations suggest that the use of synthetic media as presporulation media is preferable to that of malt wort. Synthetic media are essential for the study of the effect on sporulating ability of variations in concentration of individual constituents such as glucose.

Estimations of the glycogen content of yeast cells during fermentation have shown that storage of this polysaccharide is not necessary for sporulation; this conclusion accords with the findings of Pazonyi & Markus (1955). Storage of fat was found to be detrimental to sporulation. This is not surprising, because the formation of fat depends on a vigorous aerobic metabolism which is inimical to the promotion of sporulating ability.

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SOME STUDIES OF THE COLONY COUNT TECHNIQUE FOR SOIL BACTERIA

By J. W. EGDELL*, W. A. CUTHBERT, C. A. SCARLETT and S. B. THOMAS
National Agricultural Advisory Service
(**Ministry of Agriculture, Fisheries & Food, Westbury-on-Trym, Bristol*)

AND

M. H. WESTMACOTT
Statistics Dept., Rothamsted Experimental Station, Harpenden, Herts.

WITH THE COLLABORATION OF

ELIZABETH R. BIRD and J. HARRISON
National Agricultural Advisory Service

SUMMARY: A series of co-operative experiments was conducted to compare the bacterial colony counts of soil obtained by workers in different laboratories, using soil extract agar and other media for the determinations.

In the earlier experiments it was not possible to obtain a reasonable degree of reproducibility of results between laboratories even when the plating technique was carefully prescribed. By modification and more rigid standardization of the technique closer agreement was obtained in subsequent experiments. It is suggested that when co-operative investigations are contemplated the participating laboratories should check their technique by the examination of 'control' soils.

No evidence could be obtained to support the suggestion that higher colony counts are obtained by the use of soil extract media containing extract prepared from the same soil as the sample tested. The source appears to be immaterial so long as the soil for extract preparation is not of extreme type and has been well manured and cultivated.

Several methods, involving different media, have been used for the bacteriological examination of soils by the plating technique. The chief qualities required in the ideal plating medium are: that it should support the growth of as many as possible of the autochthonous bacteria in the soil, and that it should give reproducible results. Winogradsky (1923) observed that the majority of the bacteria in soil occur as aggregates and not as single cells, so that the plate count is likely to underestimate the total population. Conn (1914), and many other workers since, have shown that no single medium can support the growth of more than a relatively small proportion of the total soil flora. Differences in the composition of the medium produce variations in the total count obtained. Media made from natural materials may vary from batch to batch and cause variable counts, and enriched media tend to support the growth of zymogenous organisms and retard or inhibit the development of the true autochthonous soil organisms.

In order to control some of the factors producing variability in the medium itself. Thornton (1922) devised a synthetic medium made with defined materials; but though giving fairly reproducible results it is selective, and markedly lower colony counts are obtained with it than with media containing naturally occurring ingredients. Other workers have made use of non-selective media, the albumin-glucose-salts medium of Waksman (1931), for instance, which has been widely used in America. Cunningham (1947) devised a simple soil extract medium which gave higher counts than Waksman's medium, and Topping & Gibson (1936) recommended one containing Yeastrel and peptone, but not soil extract, claiming that it yielded higher counts.

With the object of determining the variability of the plating technique, a series of co-operative experiments has been carried out in laboratories at Aberystwyth, Bristol and Leeds. Laboratories at Cardiff, Reading and Starcross also took part in the final experiment. Some additional small scale experiments were carried out at one or more of these centres, at intervals during the main series.

METHODS

The technique employed in the first experiment was amended for later experiments; this applied particularly to the preparation of the soil extract media. Where the methods were different from those appended below, the variations introduced are recorded under the particular experiment.

Preparation of soil extract. Soil for soil extract was taken from the top 6 in. of an arable plot which had received dung and complete artificial manuring during the previous year, and passed through a 3 mm sieve. The sieved soil was mixed with its own weight of glass distilled water, and the mixture autoclaved for 1 hr at 15 lb/in^2. Calcium sulphate (0·5 g/l.) was added, and the suspension filtered hot through paper pulp in a Buchner funnel. After experiment 2, Whatman filter paper was used instead of paper pulp.

When soil for extract preparation had to be sent from one testing centre to another the sieved soil was placed in clean soil sample bags which were packed in sealed biscuit tins and despatched by rail without delay. The portion of soil retained at the despatch laboratory was similarly packed and stored in a cool situation until required.

Preparation of Taylor's medium (1951). The composition of 1 l. of medium was: K_2HPO_4, 0·4 g; $MgSO_4.7H_2O$, 0·05 g; NaCl, 0·1 g; $FeCl_3$, 0·01 g; Bacto peptone, 0·5 g; Difco yeast extract, 0·5 g; soil extract, 25% v/v; and agar, 15 g. To prepare 5 l. of medium, the chemicals and 1·25 l. of soil extract were added to a flask containing 3 l. of distilled water. The contents were dissolved by steaming and adjusted to pH 7·6 at 50° using phenol red as indicator. The agar was then added, the volume made up to 5 l. with distilled water, and the mixture was autoclaved at 15 lb/in^2 for 20 min and filtered hot through paper pulp. The pH was determined at this stage and readjusted if necessary to 7·0 at 50°, after which the medium was tubed in 15 ml quantities and autoclaved at 15 lb/in^2 for 20 min. Because autoclaving altered the final pH of the tubed medium used in experiment 1, for subsequent experiments about 200 ml of the bulk medium after filtering was distributed in 10 ml quantities in 6×⅝ in. test tubes, adjusted to a range of pH values, autoclaved, and the pH determined, in order to see what pre-autoclaving pH value would give a value of 7·0 in the medium after autoclaving. The bulk medium was then adjusted accordingly; this served to reduce the variation in the pH of the tubed media to 7·0±0·2.

Preparation of Skinner, Jones & Mollison's medium (1952). The medium consisted of $Ca(NO_3)_2$, 0·66 g; $CaSO_4$, 1·6 g; $MgSO_4.7H_2O$, 1·4 g; K_2SO_4, 0·05 g; $FeCl_3$, 1 ml of a 0·2% solution; glucose, 0·01 g; Bacto yeast extract, 0·005 g; and agar, 15 g; in 1 litre. The agar was placed in a 2 l. flask marked at 1 litre, covered with distilled water and allowed to soak for 1 hr. The wash water was decanted off and the washing procedure repeated twice. The salt solution (500 ml) made up with distilled water was added, the $CaSO_4$ having been ground up with a little distilled water to assist it to dissolve, and the volume made up to 1 litre with distilled water. The contents of the flask were steamed to dissolve the agar and filtered hot through paper pulp. The glucose and yeast extract were added to the filtrate, and the whole cooled to 60° and adjusted to pH 6·6. The medium was tubed in 15 ml quantities, autoclaved at 15 lb/in^2 for 20 min and the pH determined to ensure it was 7·0–7·2.

Lochhead's soil extract medium (*Lochhead* & *Thexton*, 1952). The medium was prepared by adding K_2HPO_4, 0·2 g and agar, 15 g to 1 l. of soil extract and autoclaving at 15 lb/in^2 for 20 min. After preliminary pH trials, as described for Taylor's medium, the pH of the bulk medium was adjusted to give the required pH values for the tubed medium, which was filtered hot, tubed in 15 ml quantities and autoclaved.

Collection and treatment of soil samples. Samples were taken from various soils for the experiments. They normally consisted of not less than 20 cores of soil from the top 6 in. collected in sterile wide mouthed glass jars. When soil under grass was sampled the top half inch of the core was discarded. Sufficient soil was taken so that 1–3 Kg, depending on the particular experiment, was available after sieving. The sample was brought to the laboratory within 2 hr of sampling and immediately passed through a sterile 3 mm sieve to remove stones and large roots. When it was necessary to fray the soil through the sieve with the hand, sterilized rubber gloves were worn. The sieved soil was then transferred to a clean glass plate previously swabbed with alcohol and flamed, and thoroughly mixed. A homogeneous sample for examination was obtained by repeated quartering of the heap until some 100 g remained. Of this quantity, 50 g were used for the bacteriological examination and 10 g for moisture determination. The colony counts were subsequently calculated on a dry soil basis.

When soil for examination had to be despatched from one testing centre to another a 50 g quantity of sieved soil was weighed into a sterile wide mouthed glass jar, which was sealed with a sterile rubber closure, and the jar then packed in a large wooden container lined with cork chips and transported by rail. In this way moisture and temperature changes were minimized. When, because of the nature of the experiment, examination was not carried out immediately, similar containers were used for storing soil in a cool situation in the laboratory.

Examination of samples. Fifty grammes of thoroughly mixed, sieved soil were placed in a sterile 500 ml blood transfusion bottle, 450 ml of quarter-strength Ringer's solution added and the mixture shaken vigorously by an up and down movement through an excursion of 1 ft for 5 min. This constituted the 10^{-1} dilution of moist soil. From this, 10^{-2} to 10^{-7} dilutions were made by adding 10 ml of the appropriate dilution to 90 ml of sterile quarter-strength Ringer's solution in 4 oz screwcapped bottles. A fresh, sterile pipette was used for each dilution and each bottle was shaken 10 times through an excursion of 1 ft to mix the contents. With the suspension kept in motion, three replicate plates were inoculated with 1 ml from each of the 10^{-5} and 10^{-6} dilutions. After adding the media the inverted plates were incubated in a water jacketed incubator at 26·5 ± 1·5° for 14 days, after which the colonies were counted.

Statistical treatment. The statistical treatment of the results was very similar for each experiment; the data were recorded in the form of numbers of bacterial colonies occurring on each plate. As a rule two dilutions were plated, and there were three replicate plates at each dilution, though this number was raised to four in experiment 6.

The first step in the analysis was to decide which dilution provided the best basis for comparison of the results. This was a simple decision, as there was usually only one dilution which provided results which did not contain a large proportion of zeros or a large proportion of counts of over 500/plate (300 in later experiments). As is noted in the section on this subject, comparisons between counts made at different dilutions are unlikely to be valid. The next step was to obtain log $(n+1)$ for each colony count n, in order approximately to 'normalise' the distribution. All subsequent calculations were carried out on these logarithms.

In all experiments there were a few plates which were spoiled, either because of spreading colonies or for other reasons thought not to be connected with the factors being investigated. In these cases, either the mean of the log counts of the surviving plate or plates was written in or, where appropriate, the 'missing value' technique of Yates (1933) was used to fill in the missing observation for the purposes of computation. The degrees of freedom of the corresponding error sums of squares were reduced by the numbers of missing values.

After the calculation of 'missing values' the analyses of variance were carried out, and on the completion of the analyses the means of the logarithms were calculated. These were then 'de-transformed' and multiplied by the appropriate factors for dilution number and soil moisture content.

EXPERIMENTS AND RESULTS

Experiment 1. The object of this experiment was to compare the colony counts obtained on Taylor's soil extract agar (Taylor, 1951), using media prepared from the extracts of three different soils, with those on the non-selective medium of Skinner, Jones & Mollison (1952).

Each of the three co-operating laboratories, Aberystwyth, Bristol and Leeds, prepared a quantity of Taylor's medium in test tubes and sent one-third of it to each of the other two centres. Each centre determined the bacterial populations of at least six soils using the three soil extract media, and also the non-selective medium which each centre prepared. Six soil samples were examined at Aberystwyth and

Bristol and seven at Leeds. A wide range of soils was included in the 19 samples. from very fertile arable loam to old grassland, and the pH of the samples ranged from 5·3 to 8·0.

For this experiment a countable plate was defined as one containing not more than 500 colonies. The mean colony counts, which were calculated from the 10^{-6} dilution counts, and the means of the log counts with their appropriate standard errors, are shown in Table 1. Standard errors are derived from the interactions of soils with media and are based on 18 degrees of freedom for Leeds and 15 for the other two centres.

Table 1. *A comparison of the mean colony counts of soils on Taylor's soil extract medium prepared at three centres; and on the non-selective medium of Skinner, Jones & Mollison* (*exp.* 1)

Medium	Prepared at*	Mean colony count (n) (10^6/g dry soil)† at*			Mean values of $\log_{10}$ (n+1)‡ at*		
		A	B	L	A (±0·058)§	B (±0·053)§	L (±0·037)§
Taylor's	A	8	14	21	0·96	1·17	1·35
	B	23	96	77	1·38	1·99	1·89
	L	18	68	54	1·28	1·84	1·74
Non-selective	Each centre	6	8	49	0·84	0·95	1·70
Geometric means		12	30	46	1·11	1·49	1·67

* A, Aberystwyth; B, Bristol; L, Leeds.

† Derived from log counts.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors (in brackets) are based on 15, 15 and 18 degrees of freedom at A, B and L respectively and are for vertical comparisons only. They are derived from the interactions of soils with media.

At every centre the soil extract medium prepared at Bristol gave the highest counts, followed by the Leeds medium. It was anticipated that the non-selective medium would give lower colony counts than the soil extract media; this was true at Aberystwyth and Bristol, but at Leeds the Aberystwyth soil extract medium gave the lowest counts.

The mean colony counts were lower at Aberystwyth than at the other two centres. This was no doubt due partly to the fact that all the soils tested there were under grass and of a relatively low pH. so that a lower bacterial population would be expected. But at all centres there was evidence of some interaction between soils and media; that is to say, the differences between the bacterial counts of the soils tested were not exactly the same on all media.

From the results of this experiment it could only be concluded that colony counts obtained in different laboratories using soil extract agar made up from their own soils would not be closely comparable. There was no means of telling. however. whether this was due to variations in the methods of making up the extracts or to differences between the soils. It must be recorded that in the experiment no proper randomization sequence was followed in the inoculation and pouring of the plates for the three soil extract media: there is thus the possibility that the results were disturbed by uncontrolled effects which affected the treatments unequally. However, in view of the small differences between replicates in subsequent experiments it seems unlikely that disturbances of this type did in fact have any appreciable effects. Again, it was not possible to say whether the low counts at Aberystwyth were due wholly to a low bacterial flora in the soil or due partly to differences in technique.

It was observed that, apart from a lower pH value, the Aberystwyth medium was darker in colour than those of the other two centres, and it may be that the colour was associated in some way with failure of the medium to support the growth of some organisms, resulting in lower total counts.

The results, although not altogether consistent, did indicate that it was possible to obtain higher total counts on soil extract agar than on the non-selective medium. The errors between replicate counts on the four media were of very similar size and there was no evidence that differences in the ease of counting had any effect on the accuracy of the counts. On the other hand, the colonies on the non-selective medium were smaller and there was rather less spreading growth to interfere with the counting or with subsequent 'picking' of colonies for nutritional grouping purposes.

Experiment 2. In order to throw light on the differences in the results obtained in experiment 1, each of the three co-operating laboratories prepared four batches of Taylor's soil extract agar, one at pH 7·0 and one at pH 6·0 with extract from its own soil, and two at pH 7·0 from soil supplied by the other two centres.

The soil for the making of the soil extract was sampled, sieved and despatched, or stored, on the same day at all three centres. On the day following sampling the extracts were prepared at each centre and stored in the refrigerator overnight, the media making being completed on the next day. After this the tubed media were stored at 3–5° until required.

Table 2. *A comparison of the mean colony counts of six soils at each of three centres on Taylor's soil extract medium at two pH levels, prepared from three soils* (*exp.* 2)

Centre† supplying soil for extract	pH of medium	Mean colony count (n) (10^6/g dry soil)* at†			Mean value of $\log_{10}(n+1)$‡ at †		
		A	B	L	A (±0·025)§	B (±0·031)§	L (±0·031)§
A	7·0	26	25	78	1·44	1·42	1·90
	6·0	25	—	—	1·41	—	—
B	7·0	30	42	92	1·49	1·64	1·97
	6·0	—	29	—	—	1·48	—
L	7·0	22	34	89	1·38	1·54	1·96
	6·0	—	—	98	—	—	1·99
	Geometric means	26	32	89	1·43	1·52	1·95

* Derived from log counts. Three batches at pH 7·0 and one at 6·0.

† A, Aberystwyth; B, Bristol; L, Leeds.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors (in brackets) are derived from the interactions of soils with media, with 15 degrees of freedom, and are for vertical comparisons only.

Six soils were examined at each centre, the 18 soils covering a wide variety with pH values ranging from 4·4 to 7·9. Because of the tendency to overcrowding of colonies which was revealed in experiment 1, a countable plate was regarded as one with up to 300 colonies only. The mean colony counts are presented in Table 2 in the same form as those for experiment 1.

At all three centres the medium made from the Bristol soil gave the highest counts. The differences between the media made from the Aberystwyth and Leeds soils were not consistent. Lowering the pH of the medium had very little effect at any laboratory. The differences between media in this experiment were considerably less than those recorded in experiment 1, which indicates that differences in methods of making up the extracts were probably important in the latter. Experiment 2 did show, however, that some part of the differences in experiment 1 may have been due to the soils used for the extracts.

As in experiment 1, the mean counts at Leeds were the highest, and those at Aberystwyth the lowest. This may well have been due to the nature of the soils sampled, which at Aberystwyth were chiefly from rather poor grassland and at Leeds mainly from cultivated and fertile land. There was again no proper randomization in this experiment, so that the same reservations as before must be made about uncontrolled effects. There were significant interactions between the media used and the soils tested at Leeds but not at the other two laboratories.

Experiment 3. Since Lochhead (1952) had criticized Taylor's medium on the grounds that the incorporation of tryptone and yeast extract favoured the growth of the zymogenous rather than the autochthonous flora, it was decided to compare the two media under similar conditions. Each of the three laboratories made up three batches of Taylor's and three of Lochhead's soil extract agar at pH 6·8, 6·5 and 6·0, all using soil supplied by Bristol. Each centre used the six media to determine the bacterial populations of the soils tested.

The soil for the extract was obtained from a single source and sent to the other two centres in airtight containers from Bristol. The method of preparing the soil extract was slightly modified. The quantity of soil autoclaved in each flask was limited to 1 Kg. Also, the contents of each flask were filtered hot through a no. 5 Whatman paper instead of through paper pulp, and the filtrate from each flask was made up to one litre with distilled water at 50°. Otherwise the methods of preparation were essentially similar to the original methods quoted.

After the preliminary pH trials bulk Taylor's medium was divided into three equal volumes and the preparation completed so that the three batches would have final pH values of 6·8, 6·5 and 6·0. This pH adjustment of the bulk media was not altogether successful, as is seen from the final pH values given with the mean colony count results in Table 3. It is difficult to explain the differences in pH unless they were due to instability of the media during autoclaving.

The general procedure for sampling and testing was as before, three replicates of the 10^{-5} and 10^{-6} dilutions being plated; but the 18 plates for each dilution were inoculated and poured in a randomized order and the plates for each replicate were stacked together in the incubator. Stacks of not more than three plates were made and all the plates for any one soil sample were incubated on the same shelf. When the plates were counted a record was made of any interference due to moulds and spreading bacterial colonies. Six soils, covering a wide range as before, were examined at each centre.

Table 3. *A comparison of the mean colony counts on two soil extract media made at three centres using one soil for the extract* (*exp.* 3)

Medium, with Bristol soil extract	pH and mean colony count (n) (10^6/g dry soil)* at†						Mean value of $\log_{10}$ (n+1)‡ at†		
	A		B		L		A	B	L
	pH	no.	pH	no.	pH	no.	(±0·054)§	(±0·089)§	(±0·035)§
Taylor (1951)	7·2	34	7·2	58	6·5	71	1·54	1·77	1·86
	6·8	34	6·8	64	6·5	36	1·54	1·81	1·57
	6·0	38	6·0	86	6·1	61	1·59	1·94	1·79
Lochhead & Thexton (1952)	6·8	23	7·0	80	6·6	71	1·38	1·91	1·86
	6·6	26	6·8	104	6·3	68	1·43	2·02	1·84
	6·2	30	6·5	116	6·2	56	1·49	2·07	1·76
Geometric means		31		82		59	1·50	1·92	1·78

* Derived from log counts, six soils used at each centre.

† A, Aberystwyth; B, Bristol; L, Leeds.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors (in brackets) are derived from the interactions of soils with media, with 25 degrees of freedom, and are for vertical comparisons only.

Table 3 shows that in spite of the increased control over the technique the results were still not consistent from centre to centre. Taylor's medium gave higher counts at Aberystwyth, and Lochhead's at the other two centres. The effect of the pH of the medium on the colony count was quite inconclusive because of the variable pH values. Lower counts were associated with the higher pH values at Aberystwyth and Bristol but not at Leeds, where although the recorded pH for two batches of Taylor's medium were identical the mean colony counts differed widely. There was no evidence of interactions between soils and media at Aberystwyth or at Leeds, but these were considerable at Bristol. It was clear from the results of this experiment that further efforts should be made to standardize the techniques employed. It was also felt that it would be an advantage if, in future experiments, the same soils could be tested at the different centres.

Experiment 4. From the results of experiment 3 it appeared that there was little to choose between Taylor's medium and Lochhead's medium so far as the magnitude of the total counts was concerned. As Lochhead's medium is the simpler of the two to prepare it was decided to use it in future experiments.

For this experiment, which was designed to eliminate some of the errors in the previous experiments, each of the three laboratories supplied soil for media making to the other two, and each prepared a batch of Lochhead's soil extract agar from each soil. Half of each batch was adjusted to pH 6·25 and half to pH 6·75. Subsequently each laboratory supplied a further sample of the same soil to the other two for examination, and colony counts for the three soils were obtained on the six media at each centre. Closer attention than before was paid to details of technique.

Aberystwyth sampled soil of pH 6·2, and the Bristol and Leeds soils were of pH 7·8 and 7·1 respectively. The soils for extract production were despatched to the other centres in sealed tins on the day of sampling. On the fourth day after sampling all centres made up a batch of medium from each of the three lots of soil. The preparation of the extract was as for experiment 3 except that the filtrate was not made up to 1 litre with distilled water. When the soil for extract preparation was autoclaved, the temperature was raised as quickly as possible and the time taken to reach 15 lb/in² recorded. After autoclaving, the flasks of soil were transferred to a steamer to keep hot and the contents filtered as quickly as possible through Whatman no. 5 filter paper in a Buchner funnel. The same procedure as before, using colorimetric methods, was followed in order to obtain final pH values of 6·25 and 6·75 in the media. The pH was measured before the trial autoclaving at 63° and afterwards at 26·5°.

Three days after each centre had despatched the soil for examination to the other two centres, the three soils were examined at each centre. The methods of making the dilutions were exactly prescribed and the degree of shaking in mixing the dilutions was carefully laid down. The plates were poured according to the instruction in Memo 139/Foods (Ministry of Health, 1937) and a randomized sequence followed. Care was taken to carry out the plating in the absence of direct sunlight.

The numerical results are shown in Tables 4 and 5. Since the same extracts were used and the same soils tested it was hoped that closely comparable results might be obtained, especially as efforts were made to control the laboratory technique

Table 4. *A comparison of the mean colony counts for three soils on Lochhead's soil extract agar at three centres* (*exp.* 4)

Centre	Centre† supplying soil for extract	Mean colony count (n) (10^6/g dry soil)* from†			Mean value of $\log_{10}(n+1)$‡ from†		
		Soil A	Soil B	Soil L	Soil A	Soil B	Soil L
						(±0·026)§	
Aberystwyth	A	44	101	94	1·65	2·01	1·98
	B	31	131	68	1·50	2·12	1·84
	L	40	99	64	1·61	2·00	1·81
	Geometric means	38	109	75	1·59	2·04	1·88
						(±0·084)§	
Bristol	A	62	177	158	1·80	2·25	2·20
	B	64	144	101	1·81	2·16	2·01
	L	55	161	86	1·75	2·21	1·94
	Geometric means	59	158	111	1·78	2·20	2·05
						(±0·042)§	
Leeds	A	5	12	10	0·75	1·12	1·02
	B	40	144	147	1·61	2·16	2·17
	L	9	37	26	0·98	1·58	1·44
	Geometric means	12	41	34	1·11	1·62	1·54

* Derived from log counts, the same three soils being used at each centre.

† A, Aberystwyth; B, Bristol; L, Leeds.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors (in brackets) are derived from differences between replicate plates, with 32–34 degrees of freedom, and apply to comparisons within each block of nine figures.

Table 5. *A comparison of the mean colony counts on three batches of Lochhead's medium at each of two pH levels (exp. 4)*

pH of medium	Centre† supplying soil for extract	Mean colony count (n) (10^6/g dry soil)* at†			Mean value of $\log_{10}(n+1)$‡ at†		
		A	B	L	A	B	L
					(±0·051)§	(±0·061)§	(±0·089)§
6·25	A	77	116	2	1·89	2·07	0·54
	B	71	94	99	1·86	1·98	2·00
	L	64	104	13	1·81	2·02	1·16
	Geometric means	70	104	16	1·85	2·02	1·24
					(±0·051)§	(±0·061)§	(±0·089)§
6·75	A	71	122	23	1·86	2·09	1·38
	B	59	99	90	1·78	2·00	1·96
	L	61	82	31	1·80	1·92	1·51
	Geometric means	65	99	41	1·82	2·00	1·62

* Derived from log counts, the same three soils being used at each centre.

† A, Aberystwyth; B, Bristol; L, Leeds.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors (in brackets) are derived from interactions between soils counted and media, with 10 degrees of freedom, and are for vertical comparisons only.

closely. This hope was realised at Aberystwyth and Bristol, but the Leeds results, as in the previous experiment, were anomalous. It was clear that some major factor had entered into the preparation of the Aberystwyth and Leeds soil extracts at Leeds. This anomaly was further investigated and is discussed later. Apart from the fact that the colony counts at Bristol were consistently higher than those at Aberystwyth, the results from these two centres were very similar, both in respect of differences between media and differences between soils. It is rather surprising to find that the differences between the soils at Leeds were not dissimilar to those found at the other two centres, in spite of the marked difference between media. The effects of differences in the pH of the media at Aberystwyth and Bristol were very small, but consistent with the previous experiments in that the colony counts fell slightly with rising pH.

There were significant interactions between soils and media at Aberystwyth and Leeds; at Bristol, the interactions were apparently of similar size, but as the error between replicate plates was slightly higher the interactions were not significant. It has been suggested that the bacterial count of a soil should be highest when an extract of the same soil is used for making the plating medium. If this were true, it should be reflected in the pattern of the interactions between soils and media; the figures on the diagonals of Table 4 should on the average be greater than the other figures, but there is no evidence of this. The interactions conform to no obvious pattern and it is probable that they were due to uncontrolled differences in making batches of media.

The effect of method of treating soil suspension for extract preparation. As a result of observations made during the foregoing co-operative experiments it became apparent that the process of preparing soil extract was by no means uniform in operation. The time taken to filter a given volume of soil suspension varied with the type of soil, the temperature of the suspension, the thickness of the pulp filter and the vacuum applied. Even in experiments 3 and 4, when the filter was a single no. 5 Whatman paper, it clogged up with colloidal material to such an extent that filtration was completely arrested within a very short time if the soil contained a high proportion of clay and fine silt. Lochhead, in his work, used a no. 5 Whatman filter paper. Experiments conducted with different grades of filter paper, however, showed that a no. 54 Whatman paper gave a greater volume of filtrate than a no. 5 paper, the volumes from 1 litre of suspension being 380 ml and 200 ml respectively after 20 min and 400 ml and 310 ml respectively after 80 min.

Comparing hot filtration with cold filtration, in which the suspension was allowed to cool and settle for 2 hr, in the first 20 min of filtration the former method was far more rapid than the latter, producing nearly double the volume of filtrate; but as filtration continued the amount of filtrate obtained by the cold method tended to approach that for the hot method. Further small scale experiments on the effect of allowing the fine colloidal particles to settle for various times before filtration showed that the advantages of settling were greatly diminished with clay soils. Unless a sandy loam soil is always readily available for providing extract, it does not seem that cooling and settling of the suspension is a satisfactory solution to the problem. Gibson (1953, pers. comm.) stated that the best way to filter the soil suspension is to decant the hot supernatant fluid from the flask into a separate vessel, transfer the coarse granular sediment from the flask to the filter paper in the Buchner funnel, and subsequently add the supernatant fluid to the funnel. The object of transferring the granular material first is to arrest a proportion of the colloidal material which would otherwise clog the filter paper. Several tests of this method were carried out, the results of which substantiated Gibson's statement.

From this work it was concluded that for most light and medium soils hot filtration was better than cold filtration, and that transferring the coarse granular material to the filter paper before adding the supernatant fluid, and filtering hot, gave the best results; using a sandy loam soil, some 50% of the final yield of extract was obtained in 20 min. Further, because of the difficulty of filtering clay soil a plot of fertile sandy loam soil should be reserved for obtaining soil for extract preparation, while no. 54 Whatman filter paper was superior to no. 5 paper, and to paper pulp, in rate of filtration.

The influence of different methods of filtration of the soil suspension on the bacterial colony count subsequently obtained with the soil extract media was then examined. Tests were made in which different types and thicknesses of paper pulp and unpulped filter paper, and a fine Seitz filter, were used, and centrifuging was also tried. These tests showed that the counts decreased steadily with the degree of filtration applied. Thus in one experiment, in which filtration through one, two and three layers of filter paper was compared with Seitz filtration, the mean counts of three replicate 10^{-6} dilution plates were 168, 133, 124 and 108 respectively. In other tests, paper pulp generally gave higher results than Seitz filtration, while centrifuging the suspension at 4,000 rev/min tended to give slightly higher counts than filtering through a single layer of filter paper. For convenience of standardization, filtration through a single layer of Whatman no. 54 paper was adopted.

Another factor which it was thought might partly account for the variations between laboratories was the time of autoclaving, not only the soil suspension for the soil extract but also the complete media. Although this had been specified in the technique for the co-operative experiments, different autoclaves take varying times to reach the required pressure and to return to atmospheric pressure after the process. The times necessary to reach 15 lb/in² (from 100°) in experiment 4 were recorded and it was found that at Bristol the process took only 5 min, whereas at Aberystwyth it required 4–17 min, with an average of 8 min, and at Leeds 8–16 min, with an average of 11 min.

Experiments were carried out at Bristol on the effect of various combinations of autoclaving pressure and time given to soil suspensions for soil extract preparation, and to complete agar media, on the colony counts subsequently obtained. The results of one experiment are shown in Table 6; they are typical of the data obtained. The differences between the various treatments were consistent but small, and certainly could not have accounted for the considerably larger differences which occurred between laboratories in the co-operative experiments.

Table 6. *The effect of different times and pressures of autoclaving both soil suspension for soil extract, and the complete medium, on the mean colony count of soil. Mean colony counts of* 10^{-5} *dilution plates*

Treatment of soil suspension		Treatment of complete medium (lb/in² and min)							
(lb/in²)	(min)	15		20		15		20	
		20	30	20	30	20	30	20	30
		Mean colony count (n)				Mean values of $\log_{10}(n+1)$*			
15	60	354	294	320	278	2·55	2·47	2·50	2·44
	90	360	—	—	—	2·56	—	—	—
20	60	310	—	—	—	2·49	—	—	—
	90	313	—	—	—	2·50	—	—	—

* The standard error of ±0·021 is based on differences between replicate plates and refers to all comparisons, with 12 degrees of freedom.

The effect of cotton wool extract. Before repeating co-operative experiment 4 it was decided to ascertain whether the anomalous results obtained at Leeds in that experiment could have been due to toxicity in some of the tubes of media caused by condensate from the dyed cotton wool plugs, as different coloured wools were used to identify the six media.

Concentrated extracts of cotton wool of five different colours were prepared by autoclaving 5 g of cotton wool with 150 ml of distilled water at 15 lb/in² for 20 min, decanting the extract and autoclaving it again. In the course of a soil count, 1 ml of sterile cotton wool extract was added to each plate at the same time as the soil inoculum, and two batches of soil extract agar, which had been made up on successive days, were poured. Thus there were two batches of media and five cotton wool extracts, together with distilled water controls. Each centre used its own soil for the preparation of media and for bacterial colony counts, and the details of technique were very similar to those in experiment 4.

At each centre, the media with distilled water gave slightly higher counts than those with cotton wool extracts, but the differences were far too small to account for the anomalies in experiment 4. They were in fact smaller and less consistent than the differences between the two soil extract agars, which were nominally identical, but made up on successive days. No adequate explanation of the anomalous results at Leeds was obtained.

Experiment 5. This was a repeat of experiment 4, except that the methods of filtration of the media were further modified and standardized. The chief modifications were that heavy clay soils were not used, that after autoclaving the soil suspension the water lost from the flask was replaced with hot distilled water, and that the soil suspension was gently swirled round to resuspend the fine silt and colloidal material. This fluid was then poured into a jug, which was kept warm, and the granular material at the bottom of the flask was shaken out and spread evenly over a moist no. 54 Whatman filter paper in a Buchner funnel. With the vacuum applied, the fluid was poured gently over the granular material and filtered. The same grade of filter paper, instead of paper pulp, was used for filtering the complete media.

Table 7. *A comparison of the mean colony counts for three soils on Lochhead's soil extract agar at three centres* (*exp*. 5)

Centre	Centre† supplying soil for extract	Mean colony count (n) (10^6/g dry soil)* from†			Mean value of $\log_{10}(n+1)$‡ from†		
		Soil A	Soil B	Soil L	Soil A	Soil B	Soil L
						(±0·020)§	
Aberystwyth	A	25	106	84	1·42	2·03	1·93
	B	31	137	120	1·50	2·14	2·08
	L	33	136	110	1·53	2·14	2·04
Geometric means		29	126	104	1·48	2·10	2·02
						(±0·019)§	
Bristol	A	51	155	170	1·72	2·19	2·23
	B	40	176	163	1·61	2·25	2·22
	L	47	156	140	1·68	2·21	2·15
Geometric means		46	162	157	1·67	2·21	2·20
						(±0·021)§	
Leeds	A	89	233	229	1·95	2·37	2·36
	B	80	283	259	1·91	2·45	2·42
	L	91	290	271	1·96	2·46	2·43
Geometric means		86	268	252	1·94	2·43	2·40

* Derived from log counts, the same three soils being used at each centre.

† A, Aberystwyth; B, Bristol; L, Leeds.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors (in brackets) are derived from differences between replicate plates, with 24, 14 and 32 degrees of freedom, and apply to comparisons within each block of nine figures.

The results, shown in Tables 7 and 8, were more consistent from centre to centre than those for experiment 4. The colony counts of the soils tested were ranked in the same order by each centre, although the counts at Aberystwyth showed a larger difference between the Bristol and Leeds soils than did those at the other two centres.

The differences between soil extracts were small and not significant at Bristol, slightly larger and just significant at Leeds and slightly larger again at Aberystwyth.

Table 8. *A comparison of the mean colony counts on three batches of Lochhead's medium at each of two pH levels (exp.5)*

pH of medium	Centre† supplying soil for extract	Mean colony count (n) (10^6/g dry soil)* at†			Mean values of $\log_{10}(n+1)$‡ at†		
		A	B	L	A	B	L
					(±0·020)§	(±0·043)§	(±0·029)§
6·25	A	55	104	149	1·74	2·02	2·18
	B	77	105	168	1·89	2·02	2·23
	L	77	93	177	1·89	1·98	2·25
	Geometric means	69	100	165	1·84	2·01	2·22
					(±0·020)§	(±0·043)§	(±0·029)§
6·75	A	68	118	189	1·84	2·08	2·28
	B	83	104	194	1·92	2·02	2·29
	L	82	109	210	1·92	2·04	2·32
	Geometric means	77	110	197	1·89	2·05	2·30

* Derived from log counts, the same three soils being used at each centre.

† A, Aberystwyth; B, Bristol; L, Leeds.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors (in brackets) are derived from interactions between soils counted and media, with 10 degrees of freedom, and are for vertical comparisons only.

The media at pH 6·75 gave slightly higher counts than those at pH 6·25 at all three centres. This was the opposite result to that found in previous experiments.

At Aberystwyth there appeared to be no interaction between soils tested and media, the differences between counts remaining the same whichever medium was used. At Bristol this was not the case, although the interactions were not large, but at Leeds there appeared to be an interaction between the pH of the media and the count of the soils tested.

There were substantial differences between the overall mean counts at the different centres, as there had been in experiment 4 and all previous experiments. In experiments 1 and 3 these differences might have been due to genuine differences in the bacterial populations of the soils sampled; but this explanation is not possible for experiments 4 and 5, in which the same soils were counted at all three centres. There is no means of telling from these results whether the differences were due to factors which acted on the soil dilutions which were tested, or to factors which acted on the media when they were in course of preparation. Two points may be noticed about these differences between centres. The first is that the Aberystwyth counts were lower than the Bristol counts throughout the series, which may indicate a difference in technique or a constant environmental difference rather than a random fluctuation. The second is that differences in mean count do not seem to have affected the comparison of soils tested.

Experiment 6. For this final experiment to test the reproducibility of the technique, batches of media made from two soils were exchanged between centres and colony counts made of a single soil. To widen the scope of the work three additional centres co-operated, making two groups of three centres. Thus Aberystwyth, Bristol and Leeds formed one group in which each made up Lochhead's soil extract medium from Bristol soil, sent a batch of medium to each of the other two centres and tested a sample of Bristol soil on all three media. Cardiff, Reading and Starcross formed the second group, which used Reading soil for the preparation of the soil extract medium, and they too tested the same sample of Bristol soil.

The soil for extract production was sampled and despatched to other centres as in previous experiments, and on the third day after sampling each centre prepared Lochhead's soil extract agar as in experiment 5 except that it was at only one pH level, namely pH 6·8. On the day after preparation each centre sent one-third of the batch of tubed media, plugged with white non-absorbent cotton wool, to each other centre of its group. The media were stored at 3–5° until required. The soil sample for examination was sampled in the usual manner, sieved, mixed and weighed into sterile glass jars which were sealed and despatched in insulated containers to each of the other centres. The testing of the sample was the same as before except that four replicates of the 10^{-5} and 10^{-6} dilutions were made.

Table 9. *A comparison of the mean colony counts of a single soil sample on Lochhead's soil extract agar made from two soils at two groups of three different centres* (*exp.* 6)

Centre	Centre† supplying soil for extract	Mean colony count (n) (10^6/g dry soil)*, medium made at†			Geometric mean	Standard error§	Degrees of freedom	Mean value of $\log_{10}$ (n+1)‡, medium made at†			Geometric mean
		A	B	L				A	B	L	
A	B	210	209	238	219	±0·014	4	2·16	2·16	2·21	2·17
B		199	203	261	219	±0·023	6	2·13	2·14	2·25	2·17
L		170	171	176	172	±0·022	6	2·06	2·07	2·08	2·07
Geometric means		192	194	222	202	±0·017	4	2·12	2·12	2·18	2·14
		C	R	S							
C	R	223	186	194	200	±0·030	5	2·18	2·10	2·12	2·14
R		143	125	130	132	±0·027	6	1·99	1·93	1·95	1·96
S		179	191	211	193	±0·037	4	2·09	2·11	2·16	2·12
Geometric means		179	164	175	172	±0·022	4	2·09	2·05	2·08	2·07

* Derived from log counts, Bristol soil being used throughout, medium at pH 6·8.

† A, Aberystwyth; B, Bristol; L, Leeds; C, Cardiff; R, Reading; S, Starcross.

‡ Adjusted for dilution and soil moisture to correspond with colony counts.

§ Standard errors for individual centres are derived from differences between replicate plates, and those for means from interactions between centres and media, and they are for horizontal comparisons only.

The results, given in Table 9 for each group separately, show that there was reasonably close agreement between the colony counts obtained at different laboratories, except perhaps at Reading where the counts were on a slightly lower level. The differences between media were small, although they were just large enough to be significant when compared with the plating error.

DISCUSSION

The object of the series of trials reported here was to establish a technique for determining the colony count of soil bacteria on soil extract agar which would give reproducible results in different laboratories. Complete reproducibility was not to be expected in a method which involves a long chain of operations difficult to specify in every detail, and the use of variable materials.

There are numerous factors and sources of error which must enter into any comparison of results in different laboratories. Those affecting the soil sample for examination include errors due to sampling and treatment of the soil, variations in methods of preparing soil dilutions, and errors of sampling the soil dilutions for the inoculation of plates.

Errors due to sampling and treatment of the soil were reduced as far as possible by standardization of the methods employed. A detailed technique of preparing the soil dilutions was carefully laid down and followed but it is likely that there were small differences between laboratories and even between dilutions made on two occasions in the same laboratory. Such differences could affect the comparisons between laboratories in experiment 5 and the comparisons between soils tested within the laboratories in experiments 3 and 4.

Under ideal conditions, the numbers of cells capable of forming colonies and contained in a quantity of soil suspension can be expected to conform to a Poisson distribution, and the variance between replicate samples will be equal to the mean colony count. Such conditions are seldom met with in practice. If there is antagonism or competition between neighbouring organisms the error will tend to be less than the Poisson error. If there is clumping and bunching of the cells it will be greater. Differences in numbers of cells adhering to pipettes, and counting and other errors, will tend to increase the variance between replicate plates. In the present series of experiments the error between replicate plates was on the average somewhat smaller than the expected Poisson error.

A second category of effects consists of those affecting the media used for plate counts. These include those due to the choice of the soil for extract preparation, variations in the quality of other constituents of the medium and variations in methods of preparation of the medium.

In experiment 2 the Bristol soil gave the highest counts, which were on the average 30% higher than those on the Aberystwyth soil extract medium. The differences between soil extracts in experiments 4 and 5 were much smaller, apart from the aberrant results at Leeds, and there was also little difference between the soil extracts made from soils supplied by Bristol and Reading in experiment 6. The number of different soils used for media in this series of experiments was not large, but it seems likely that, provided a fairly light fertile soil is used, the actual choice of soil is immaterial.

There were no tests of differences between other constituents of the medium in the co-operative experiments, but since in these a particular brand of agar was not specified a small scale experiment was conducted at Bristol to determine whether different brands of agar could have caused differences in the colony counts obtained in different laboratories when they prepared their own media. Three brands of agar were compared, but there were no marked differences in the soil colony counts obtained. It is possible, however, that differences between different brands of agar could be important, and the brand should be specified when co-operative work is carried out in different laboratories.

Variations in methods of preparation of the medium seem to be the most important of the effects or sources of error so far considered. We have discussed above the influence on the total count of methods of filtration and of treatment in the autoclave. The effects of adjustment of pH in the range 6·0–7·0 were also tested in experiments 2, 3, 4 and 5. These effects were small and were not consistent from experiment to experiment or from centre to centre, but in several cases they were greater than could be accounted for by random errors due to differences between replicate plates or interactions between soils and media.

The final group of effects and errors comprises those associated with the interactions of soils and media. As mentioned above, we have found no evidence that the use of a particular soil extract gives higher counts for that particular soil than for any other soil. Nor was it found that the pH of the soil extract medium within the range 6·0–7·0 interacted with the pH of the soils examined. In the majority of the experiments reported here, the interactions of soils tested and media used were significant when compared with the differences between replicate plates, but there was no pattern to these interactions that could be detected and they had, therefore, to be regarded as 'error'.

During the later experiments an opportunity was afforded to compare the colony counts obtained for the 10^{-5} and 10^{-6} dilutions for some samples. This was only possible when the plates for both dilutions were countable, i.e. having not more than 300 colonies. From the data obtained the colony count for the higher dilution, expressed as colonies/g dry soil, was approximately $1\frac{1}{2}$ to 2 times greater than for the lower dilution. Because of the small number of samples involved it is not possible to say whether the same degree of difference applies generally to most soils, but unless colony counts can be adjusted by the application of a reliable factor, the results from different samples cannot be compared when the colony counts have been derived from different dilution levels.

Despite all the factors and errors that may affect the total plate count, there is one reassuring fact. In spite of the differences between media and the interactions between the media and soils tested, the differences between the soils tested in the various experiments were relatively constant over all the media. This indicates that differences between soils tested at the same time on the same batch of medium may be reliable, even if comparisons between different laboratories or different occasions at the same laboratory are not. It also suggests that one method of ensuring that a comparison between a number of laboratories is valid may be to distribute samples of one or more 'control' soils for plating and counting at the same time and on the same medium as the test soils.

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M. H. Westmacott is now with Shell International Petroleum Co. Ltd.

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STUDIES OF THE GROWTH OF *PENICILLIUM CHRYSOGENUM* IN CONTINUOUS FLOW CULTURE WITH REFERENCE TO PENICILLIN PRODUCTION

BY S. J. PIRT AND D. S. CALLOW

Microbiological Research Establishment,
Porton, Wiltshire

SUMMARY: A filamentous mould was cultured by the continuous flow method in which medium is supplied at a constant rate and the culture volume is kept constant. Flow rates up to 0·1 culture volumes/hr were used. The mycelial dry weight concentration and the yield of mycelium/g of carbon source used were equal to or slightly greater than the maximum obtained in batch culture. With glucose concentrations up to 80 g/l. at a flow rate of 0·05 culture volumes/hr, about 45% of the substrate carbon was converted into mycelial carbon and the remainder oxidized to CO_2.

With unlimited amounts of all nutrients available growth of the mould followed the exponential law, as does bacterial growth, and therefore the mould had a constant doubling time.

The oxygen demand of the mould as a function of growth rate was determined.

Conditions were found under which the rate of penicillin production/g of mycelium remained at its maximum value for 1000 hr.

MOULD fermentations are carried out either by the submerged batch culture method, as in penicillin production, or by the more primitive surface culture method, as in citric acid production. In the reported attempts to develop the continuous flow culture of filamentous moulds (Kolachov & Schneider, 1952; Bartlett & Gerhardt, 1958) the organism used was *Penicillium chrysogenum* and attention was concentrated on the penicillin production aspect to the exclusion of growth studies. These investigations did not show that the continuous process had any marked advantage over the batch process.

In the last decade, the continuous flow culture of bacteria has become firmly established as a research tool because of the much greater control of the environment which it makes possible (for example, see Málek, 1958; Herbert, 1958; Pirt & Callow, 1959*c*). A characteristic of these studies of continuous flow bacterial culture was the attention paid to the fundamental aspects. So far, no fundamental study of the continuous flow culture of a filamentous mould has been reported; it is the aim of this work to furnish such a study and to demonstrate that continuous flow culture according to the Monod-Novick-Szilard principles (Herbert, 1958) is applicable to a filamentous mould.

The process of penicillin production has two aspects; the first is growth of the mould, and the second is synthesis of penicillin. In fact, the process may be a two-stage one in which the conditions required for growth are different from the conditions required for penicillin formation. The work described here, in contrast to the previous investigations, was primarily on the growth of the mould in continuous flow culture. A preliminary account of this work has been given (Pirt & Callow, 1959*a*).

METHODS

Apparatus. The culture apparatus was an improved form of that described by Elsworth, Meakin, Pirt & Capell (1956). The automatic pH control system described by Callow & Pirt (1956) was used.

Aeration and agitation. Vortex aeration (Chain *et al.* 1952) was used for most of the work because the absence of baffles ensured that the walls of the vessel below the liquid level were swept clean and the accretion of mycelium on the walls prevented. Stirrer speeds in the vortex system were in the range 1000–1400 rev/min. The 63 mm diameter impeller was fitted the reverse way to that used in the baffled system, that is, with the greater height of vane above the disc. The culture volume was 1·5–2·0 l.

The culture was supplied with an excess of oxygen. This was verified by varying the oxygen demand of the culture and showing that the oxygen uptake rate was limited by the oxygen demand of the culture and not by the oxygen solution rate. This condition was in general realized when the oxygen solution rate (σ_s), determined by the sulphite method with a copper catalyst, was three times the oxygen demand of the culture (σ_c). The minimum possible ratio of $\sigma_s : \sigma_c$ was not determined.

Media. The media were based on that of Jarvis & Johnson (1947). Glucose was autoclaved separately and added at a concentration 2·3 times the dry weight required. All quantities given are in g/l.

Medium M3, used to produce mycelial dry weight up to 10 g/l., contained: $MgSO_4.7H_2O$, 0·25; $FeSO_4.7H_2O$, 0·10; $CuSO_4.5H_2O$, 0·005; $ZnSO_4.7H_2O$, 0·02; Na_2SO_4, 0·50; $MnSO_4.4H_2O$, 0·024; $CaCl_2.6H_2O$, 0·075; EDTA, 0·566; phenylacetic acid, 1·0; KH_2PO_4, 2·0; $(NH_4)_2SO_4$, 6·15. The phosphate and ammonium sulphate were mixed, adjusted to pH 6·4 with NaOH and autoclaved separately. The other salts were adjusted to pH 7·4 with NaOH and sterilized by autoclaving. There was no salt precipitation in this medium.

Medium M16, used to produce mycelial dry weight from 10 to 20 g/l., contained: $MgSO_4.7H_2O$, 0·40; EDTA, 0·744; $(NH_4)_2SO_4$, 16·95; and the other constituents of M3.

Medium M18, used to produce 20 to 30 g of dry mycelium/l., contained: $MgSO_4.7H_2O$, 0·80; EDTA, 0·982; $(NH_4)_2SO_4$, 17·8; $(NH_4)_2HPO_4$, 2·0; and the other constituents of M3 except the Na_2SO_4.

Medium M22, for dry weights between 20 and 30 g/l., contained: $MgSO_4.7H_2O$, 0·80; EDTA, 0·982; $(NH_4)_2HPO_4$, 2·0; $(NH_4)_2SO_4$, 17·8; Mo, V, Ga and Sc, each at a concentration of 0·02 mg of metal/l., the Mo being added as ammonium molybdate, V as vanadium pentoxide, Ga as the metal dissolved in HCl, Sc as ScO dissolved in H_2SO_4; and the other constituents of M3, with the exception of the Na_2SO_4.

Either 2N or 4N NaOH and 0·5N or N H_2SO_4 were used in the pH control.

The medium for growing the inoculum differed from M3 in that the phosphate was replaced by 0·1M potassium phosphate buffer, pH 6·8; the glucose concentration was 10 g/l. and phenylacetate was omitted.

Alkaterge-C (Commercial Solvents Corp., Terre Haute, U.S.A.) 30% (v/v) in liquid paraffin was added periodically to the culture to inhibit foaming (Pirt & Callow, 1958*a*).

Analytical methods. Mycelial dry weight was determined by filtering 10 ml of culture in a tared sintered glass crucible of porosity 2; the mycelial mat was washed with water and dried to constant weight at 100–105°.

Mycelial carbon was estimated from the dry weight by multiplying by the factor 0·471 (a series of carbon analyses by dry combustion of mycelium grown at different pH values gave carbon percentages varying progressively from 46·1 at pH 6·0 to 48·3 at pH 7·5).

Glucose was determined by ferricyanide reduction. Initially the samples were simply diluted to contain less than 2·5 mg of glucose/5 ml. Latterly, however, the ion exchange resin treatment of Strange, Dark & Ness (1955) was applied to samples from the culture to remove interfering substances; this treatment, it was found, removed reducing material corresponding to about 0·5 mg of glucose/ml. Samples of the culture fluid (5 ml) were shaken with 0·5 g each of acidic and basic resins.

Penicillin was determined by the biological assay method of Humphrey & Lightbown (1952); the amounts are expressed as Oxford units.

Mould strains. The strains of *Pencillium chrysogenum* used were: Wis 47-1564, Wis 49-133, Wis 54-1255, and strain D, a variant derived from Wis 51-20.

Preparation of inoculum. Stock cultures were maintained on ground maize moistened with asparagine and glycerol (Whiffen & Savage, 1947). The inoculum medium in 100 ml amounts contained in 1 l. conical flasks was given a heavy inoculum of spores, enough to ensure filamentous growth, and incubated at 25° on a shaker which gave an oxygen solution rate in sodium sulphite + copper ions of about 40 m-mole O_2/l./hr. After 40 hr growth the inoculum (200 ml) was transferred to the continuous culture vessel. At this stage the inoculum had a dry weight of 3–4 mg/ml. If phenylacetate was present in the medium to which the inoculum was transferred the pH was adjusted to 7·0; in the absence of phenylacetate the pH value could be lower.

Tests for contamination. Apart from direct microscopical examination of the culture, contaminating organisms were detected by the following tests: by plating on meat tryptic digest agar incubated at 37°; by plating on the medium of Backus & Stauffer (1955) incubated at 25°; by adding 1 ml of culture to 10 ml of meat tryptic digest broth incubated at 25°. Contamination has not been a problem in this work and no more than weekly tests were required.

Temperature. The culture temperature, unless otherwise stated, was 25°.

Other methods. Methods other than those reported here have been previously described by Pirt & Callow (1958*b*).

RESULTS

Steady state dry weight and glucose concentrations

Fig. 1 shows the dry weight at various times in cultures in which glucose supply was the growth limiting factor. The medium flow was started after an initial period of batchwise growth. The dry weight obtained in a continuous culture with a dilution rate of about 0·05 hr^{-1} was slightly higher than the maximum obtained in batch culture with the same medium. When the steady state had been reached the dry weight generally showed little variation. Factors which can cause large variation in the dry weight are: accretion of mycelium on the walls of the vessel, the dry weight increasing should mycelium fall back into the culture; formation of the pellet type of growth (Pirt & Callow, 1959*b*), causing erratic variation in dry weight; and accretion of mycelium at the effluent point, forming a filter which causes mycelium to accumulate in the vessel. This may occur if the overflow pipe is horizontal rather than vertical.

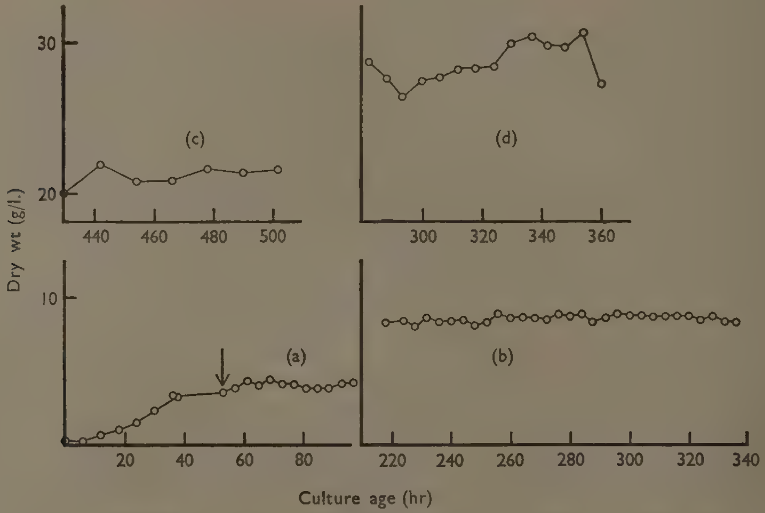

Fig. 1. Mycelial dry weights in the steady states of continuous cultures of *P. chrysogenum*. (a) Batchwise growth followed by continuous flow culture of strain Wis 47-1564 in Medium M3 (without phenylacetate) at pH 6·5, dilution rate (D), 0·086 hr^{-1}; the arrow indicates when flow began: (b) strain Wis 47-1564 in medium M3 with 19·5 g of glucose/l., pH 7·2, D, 0·079 hr^{-1}: (c) strain Wis 54-1255 in medium M16 with 54·5 g of glucose/l., pH 6·9, D, 0·055 hr^{-1}: (d) strain Wis 54-1255 in medium M18 with 82·7 g of glucose/l., pH 6·7, D, 0·057 hr^{-1}.

The concentration of residual glucose in the steady state was about 0·5 g/l., when the initial glucose concentration was 20 g/l. The residual glucose concentration increased to 1·2 g/l. when the initial glucose concentration was 85 g/l. The actual value of the residual glucose concentration may have been lower, since it was near the lower limit of the range of glucose concentration which could be estimated by the method used and also the reducing power may not have been entirely due to glucose. The results show that in the steady state of a continuous flow culture the glucose utilization may be practically complete.

The phenylacetate added did not affect the dry weight, neither did it show any toxic effect at pH values down to 6·0 in continuous flow cultures in the steady state.

However, phenylacetate seemed to be strongly inhibitory to initial growth when the culture was inoculated at pH 6·7, but not at pH 7·0.

Of the two media, M18 and M22, used to produce 30 g mycelial dry wt/l., the simpler one, M18, seemed adequate. A feature of the use of M18, as Fig. 1 shows, was that the mycelial dry weight varied much more than with the more dilute media.

Growth rate

The maximum growth rate of the mould must be known so that one can predict the maximum possible dilution rate. For continuous flow culture the growth rate is most conveniently expressed as the specific growth rate $\mu = \frac{1}{x}\frac{\mathrm{d}x}{\mathrm{d}t}$, where x is the organism concentration and t the time. If growth occurs at a constant exponential rate then μ is constant and independent of x. There seems to be a concensus of opinion that moulds do not grow at a constant exponential rate as do bacteria but that the rate of increase becomes linear after a time (Brown, 1923; Hawker, 1950). Clearly, as judged by the rate of growth of a colony on a surface, a commonly used method (e.g. Brown, 1923), a constant exponential rate of increase is not achieved; but the results of Smith (1924), who studied the growth of a single hyphal system, that is, the increase in length of the parent hypha and all its branches, show that growth at a constant exponential rate does occur.

We found that under appropriate conditions, i.e. with an unlimited supply of nutrients, constant physical environment, and no entry of toxic substances into the system, growth followed the exponential law $\frac{\mathrm{d}x}{\mathrm{d}t} = \mu x$, where μ is constant. The necessary conditions were realized in batch cultures. This is shown by the straight line obtained on plotting the logarithm of the dry weight against time (Fig. 2). The slope of the line gives a maximum specific growth rate (μ_m) of 0·075 hr^{-1}. Therefore the doubling time (t_d) was 9·2 hr. This was the doubling time observed during the initial batchwise growth following inoculation.

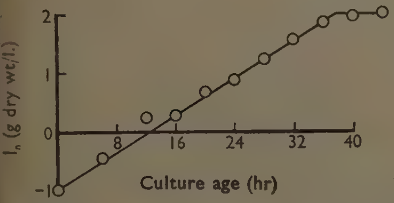

Fig. 2. Changes in the logarithm of the dry weight/l. with time after inoculation in a batch culture of strain Wis 47-1564. Medium M3 (without phenylacetate), with 20 g of glucose/l., pH 6·7.

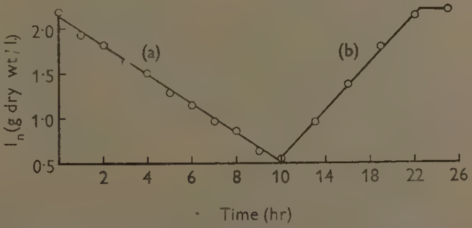

Fig. 3. Changes in the logarithm of the dry weight/l. with time in a continuous culture of strain Wis 47-1564 in medium M3, with 20 g of glucose/l., pH 7·4: (a) with dilution rate (0·296 hr^{-1}) greater than the maximum specific growth rate; (b) after stopping the medium flow.

Alternatively one can determine the maximum growth rate by a continuous flow method. If the growth in continuous culture is in accordance with the theory of Monod and of Novick & Szilard and the dilution rate (D) is increased until the organism is flowing out of the culture faster than it is being produced, that is $D > \mu_m$, an excess of nutrients should be available and the organisms should grow at the maximum rate. Under this condition the logarithm of the dry weight plotted against time should be a straight line with a negative slope equal to $\mu_m - D$, and from this by inserting D one obtains μ_m. A plot of log dry weight against time for a continuous flow culture with D about twice μ_m is shown in Fig. 3. This dilution method gave a value of 0·136 hr^{-1} for μ_m. When the flow was stopped and the culture allowed to grow batchwise the slope of the logarithmic plot shown in Fig. 3 gave the value of 0·139 for μ_m, which is in very good agreement with that obtained in the dilution method. The good agreement between the two methods was confirmed many times. The results given in Fig. 3 were obtained with a culture which had been kept for over 1000 hr in continuous culture. The much faster growth rate obtained then (doubling time 5·1 hr) compared with that in the initial batchwise growth shown in Fig. 2 (doubling time 9·2 hr) is attributed partly to the pH difference and partly to better adaptation to the environment.

Influence of pH and temperature. Maximum growth rates were generally determined by the dilution method and confirmed by subsequent batchwise growth. The influence of pH on growth rate is depicted in Fig. 4. The peak growth rate occurred between pH 7·0 and 7·4, the upper limit for growth being about 8·0. There was considerable variation in the maximum growth rate at any given pH value, as Fig. 4 shows. The variation in growth rate was well outside the experimental error, which was estimated as less than 10%. The cause of this variation may be that after any environmental change, even an apparently small one like a change in pH from 6·5 to 7·0, the organism has to be grown for a long time in the new environment before it is fully adapted and able to show its true maximum growth rate. Investigation of this effect was beyond the scope of this work.

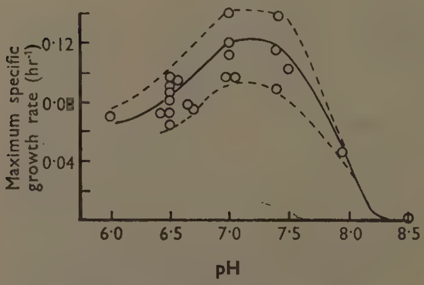

Fig. 4. The effect of pH on the growth rate of strain Wis 47-1564, in medium M3 with 20 g of glucose/l. The broken lines indicate the range of the results.

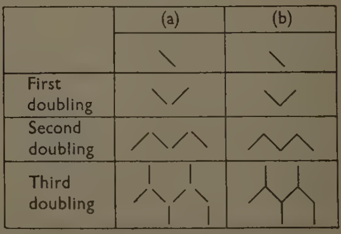

Fig. 5. A diagrammatic comparison of bacteria-like growth accompanied by fission (a) with filamentous mould growth (b).

The effect of temperature over the range 23–32° on the growth rate at pH 7·4 was investigated. There were small but not significant decreases in the maximum specific growth rate at both ends of the range.

Both pH and temperature changes, it should be noted, were made gradually, generally 0·1 pH units or 1° at a time with 2 hr intervals, as a precaution against the shock to the organism which was noted in the continuous flow culture of bacteria (Pirt & Callow, 1958*b*).

Morphology

The growth of a filamentous mould such as *P. chrysogenum* is apical and not naturally accompanied by fission, as occurs with bacteria. The two types of growth are represented in Fig. 5. Whereas growth from a single bacterial organism should in 3 doubling times produce 8 organisms, filamentous growth should result in an eightfold increase in the length of the parent hypha and its branches. If culture of the filamentous organism were indefinitely prolonged, e.g. by continuous culture, it seems that the size of the mould fragment should increase indefinitely. However, it was found that in a continuous culture in the steady state the number of individual mould fragments was roughly constant (of the order of 10^6/ml with 1% dry weight at pH 7); hence fission of the mould must have occurred. Sporulation was not responsible for the increase in the number of fragments, and it was concluded that scission of the mould hyphae was caused by the shearing action of the stirrer. Elsewhere (Pirt & Callow, 1959*b*) we have shown that the morphology of the mould depends on the pH value and the nutrition during growth.

Yield of mycelium

The weight of organism produced per unit weight of carbon source used is of considerable importance in the quantitative formulation of growth processes. Monod (1942) showed that if a bacterial population were limited solely by the amount of carbon source available, then the weight of organism produced is directly proportional to the weight of carbon source utilized, a finding which he formulated in the important equation $x = \mathrm{Y}\Delta s$, where x is the dry weight of organism produced, Δs is the amount of carbon source utilized and Y is a constant. Bacteriologists (e.g. Herbert, Elsworth & Telling, 1956) generally call Y the 'yield constant' and mycologists have called it the 'economic coefficient' (see Foster, 1949): the latter term seems to have considerable priority.

We found that the economic coefficient for *P. chrysogenum* in continuous flow cultures with excess of available oxygen was constant over a wide range of conditions. For strain Wis 47-1564 its average value (g dry wt/g of glucose utilized) was 0·443 (with standard deviation 0·043) over a wide range of conditions. This value is about the maximum reported for moulds. The coefficient was independent of the amount of glucose utilized over a wide range (up to 80 g/l.), of pH in the range 6·0–7·5, of temperature in the range 23–32°, of specific growth rate in the range 0·04–0·08 hr^{-1}, and of the strain used.

Oxygen demand

Calculations of the oxygen demands of cultures have been based on Q_{O_2} measurements (e.g. Rolinson, 1952) carried out on samples not under actual fermentation conditions and with no account being taken of growth rate. The calculated demands, therefore, may be very far removed from the true ones. Pirt (1957) showed that the oxygen demand, R_0 (m-moles O_2/l./hr) of a growing culture can be expressed as

$$R_0 = P\,\mu\Delta s$$

where P is the oxygen demand constant (moles O_2 consumed/mole substrate carbon used), μ is the specific growth rate and Δs the consumption of substrate carbon (m-mole/l.). P is characteristic of the organism and the conditions. However, it will be seen that P must lie between 0 and 1 if the carbon source has the empirical formula CH_2O, e.g. carbohydrate, and this enables one to make rough estimates of the oxygen demand and to set precise limits to it. For a continuous culture in the steady state one can substitute dilution rate for μ.

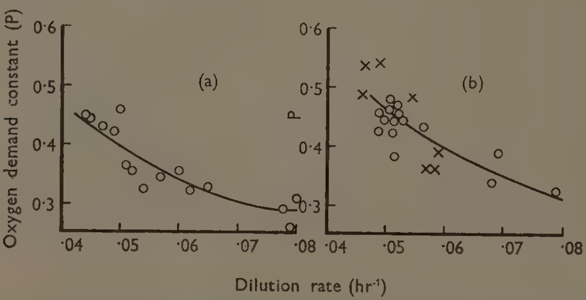

Fig. 6. The variation of the oxygen demand constant (P, moles of oxygen consumed/mole of glucose carbon) of *P. chrysogenum* with dilution rate when there was an excess of available oxygen, for (a) strain Wis 47-1564, (b) strain Wis 54-1255. Circles, at pH 7·4; crosses, at pH 6·7–7·0.

The oxygen uptake rates at various dilution rates were determined by analysis of oxygen in the effluent gas from the culture. From the values obtained, together with determination of the glucose utilized, the values of P were calculated. The variation of P with dilution rate when there was an excess of available oxygen is shown in Fig. 6. P decreased with increase in growth rate. In the middle of the growth rate range P was about 0·4, which is about the same as the values reported for bacterial and yeast species (Pirt, 1957). Variations of pH in the range 6·0–7·5 and temperature in the range 23–32° did not appreciably affect the value of P. There was a difference between the mean oxygen demands of strains Wis 47-1564 and Wis 54-1255, e.g. with D at 0·050–0·055, where most of the data were obtained, the mean value of P was 0·39 for Wis 47-1564 and 0·45 for Wis 54-1255. The maximum oxygen uptake rate observed was 76 m-mole/l./hr with a dry weight of 33 g/l.

The ratio, moles CO_2 produced: moles oxygen consumed was 1·1±0·1 under practically all the conditions used.

Carbon balances

Mycelium, carbon dioxide and penicillin were the only products determined. Table 1 shows some typical carbon balances obtained. The carbon in the mycelium and carbon dioxide accounted for virtually all the glucose carbon utilized at dilution rates up to 0·06 hr^{-1}, but at higher dilution rates, with strain Wis 47-1564, a significant part of the carbon utilized (15% with D at 0·08) was unaccounted for and may have been converted to unknown products. This behaviour resembles that of an *Aerobacter* species (Pirt, 1957), which at low growth rates and under fully aerobic conditions converts glucose entirely to CO_2 and cell carbon, but at growth rates near the maximum produces some organic acid. Yeast (Maxon & Johnson, 1953) shows analogous behaviour, but producing ethanol instead of an organic acid.

Table 1. *Carbon balances in continuous flow cultures of* P. chrysogenum *with different dilution rates*

Dilution rate (hr^{-1})	Glucose supplied (g/l.)	Glucose utilized (g/l.)	Carbon recovered* in		
			CO_2	Mycelium	Total
Strain Wis 47-1564†					
0·049	19·9	19·3	41·2	54·9	96·1
0·052	20·0	19·4	43·4	54·3	97·7
0·057	20·0	19·3	43·6	54·7	98·3
0·060	20·0	19·3	43·2	53·0	96·2
0·079	20·4	19 9	31·2	49·7	80·9
Strain D‡					
0·042	21·4	21·0	44·1	50·4	94·5
0·061	80·2	78·4	43·8	48·3	92·1

* As percentage of carbon in glucose utilized. † In medium M3, pH 7·4.
‡ In medium M3 with 21·4 g of glucose/l. and in medium M22 with 80·2 g of glucose/l., pH 6·7.

Penicillin production

The concentration of penicillin reached in a fermentation is proportional to: the mycelial dry weight concentration (x, mg/ml); the residence time (hr) of the mould in the culture, which in the case of a continuous flow culture is the reciprocal of the dilution rate (D); and to the metabolic quotient, q_{pen}, i.e. the number of units of penicillin produced/hr/g dry weight. Thus,

$$\text{Penicillin concn. (units/ml)} = \frac{x q_{pen}}{D}$$

Table 2 shows penicillin production by strain Wis 47-1564 in a continuous flow culture, and it can be seen that both the dilution rate and the dry weight may be varied considerably without significantly affecting q_{pen}. Since x and D can be varied at will, the factor determining penicillin concentration which is characteristic of the organism is q_{pen}, which is clearly an important parameter in the selection of an organism for the continuous process.

The fact that q_{pen} was stable throughout a culture which lasted nearly 1000 hr means that selection of lower yielding variants did not occur. This result is in marked contrast to that in batch cultures, in which q_{pen} falls practically to zero

Table 2. *The relationship between* q_{pen},* *culture age and dilution rate in a continuous flow culture of* P. chrysogenum†

Culture age (hr)	Dilution rate (hr^{-1})	Dry weight (mg/ml)	Penicillin concn. (units/ml)	q_{pen}*
Medium M3; glucose supplied, 19 g/l.				
120	·080	2·71	27	0·80
312	·051	8·37	175	1·07
504	·050	8·63	166	0·96
815	·046	7·58	146	0·89
Medium M16; glucose supplied, 38 g/l.				
959	·048	14·7	280	0·92

* Rate of penicillin production in units/hr/mg dry weight of mycelium.

† Strain Wis 47-1564; at 25° and pH 7·4.

Table 3. *A comparison of penicillin production in batch and single-stage continuous flow cultures of* P. chrysogenum *strain* Wis 47-1564

Reference	Nitrogen and carbon sources	Duration of culture or residence time in culture (hr)	Maximum penicillin concn. (units/ml)	Maximum rate of penicillin accumulation (units/hr/ml)
Batch culture				
Pirt (unpublished)	NH_4^+, glucose, lactose, acetate, lactate	100	300	3
Anderson *et al.* (1953)	Corn steep, lactose	90	1000	11
Continuous flow culture				
This paper	NH_4^+, glucose	20	280	14

in 100–150 hr. A comparison of penicillin production in batch culture and in continuous flow culture is given in Table 3. The former had the highest rate of accumulation of penicillin, but the penicillin concentration was lowest in the continuous culture owing to the short residence time of the mould therein.

In several cultures in which the conditions differed from those given in Table 2 an appreciable fall in q_{pen}, which was attributed to selection of lower yielding variants of the mould, was observed.

DISCUSSION

This work shows that the Monod-Novick-Szilard continuous flow culture principle, first developed for bacteria, applies equally to a filamentous mould. The practical upper limit of the flow rate with a *P. chrysogenum* culture was 0·1 culture volumes/hr.

The great advantage of the continuous flow technique is that it permits much closer control of the environment of the organism; it should therefore greatly facilitate study of the effect of environment on the mould. An example of this is the elucidation by Pirt & Callow (1959*b*) of the effect of pH and nutrition on the morphology of *P. chrysogenum* in submerged culture. In the work described here the growth rate, oxygen demand, yield of mycelium and penicillin production with much improved environmental control are reported.

The stability of the penicillin production rate over a long period and its relatively high value in continuous flow culture are noteworthy; this is another advantage which the continuous flow process possesses over batch culture. The production of a high penicillin concentration will require means of increasing the residence time of the mould in the culture without causing either a fall in the rate of penicillin production/g of mycelium (q_{pen}) or selection of lower yielding strains of the mould, conditions which, we expect, will require a two-stage process (Pirt & Callow, 1959*c*). This aspect is now under investigation.

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THE BEHAVIOUR OF INDIVIDUAL ORGANISMS IN THE LAG PHASE AND THE FORMATION OF SMALL POPULATIONS OF *ESCHERICHIA COLI*

BY L. B. QUESNEL

Department of Bacteriology, University of Bristol

SUMMARY: The generation times of individual *Escherichia coli* cells inoculated on to a cellophane membrane and supplied with a liquid synthetic medium were determined by direct observation for four generations. Viability increased and generation time decreased with increasing age of population.

IN CONSIDERING growth Hinshelwood (1946) wrote: "Bacteriologists have usually been content for their own purposes to record qualitative observations about the total growth after a standard time, a method which leaves one in doubt whether lag, growth rate, or total population have been the major factors involved." This was due largely to the absence of techniques suitable for observing undisturbed organisms during growth. However, Kelly & Rahn (1932), using agar block cultures, had been able to study the behaviour of individual cells of *Bacterium aerogenes* and *Bacillus cereus*, and on the basis of their results Rahn (1932) and Kendall (1948, 1952) presented mathematical models attempting to define bacterial growth. Later, Powell (1956*a*) developed a continuous culture chamber and used it for extensive studies on the behaviour of various organisms in the logarithmic phase of growth (Powell, 1955, 1956*b*, 1958).

To estimate the proportion of non-viable organisms occurring in bacterial populations, Wilson (1922) used classical counting techniques, Valentine & Bradfield (1954) used a urea method, and Powell (1958) made direct observations in his culture chamber. In this investigation, Powell's chamber has been used to study the lag phase of *Escherichia coli* cells.

METHODS

Apparatus. The organisms were grown under the microscope in the improved culture chamber of Powell (1956*a*), which consists essentially of a stretched cellophane membrane under which medium is circulated. In the present experiments a reciprocating micropump (MK 2, 0–375 ml capacity, Distillers Co., Ltd.) was used to circulate the medium at the rate of 40–50 ml/hr, and observations were made at a magnification of ×1500 with transmitted phase contrast illumination. The culture chamber was sterilized by autoclaving before use, but the membrane (Film PT.300, British Cellophane Co., Ltd.) was not sterilized. Powell provided a slight positive pressure in the medium, which produced an upward curvature of the membrane and held the organisms against the cover glass. In these experiments a downward curvature was used, because, once settled, the organisms generally remained in the field of observation.

Bigger's medium (Bigger, 1950), containing buffer, glucose and ammonium salts, at pH 7·4, was used. The test organisms grew well in it and maintained constant cultural characteristics on daily subculture. The strain was maintained on Dorset's egg medium, from which subcultures were taken when necessary.

Organism. This was a strain of *Escherichia coli* (β-type, 7/5/4/1) maintained in this laboratory.

Inoculation of the membrane. One drop (*c.* 0·01 ml) of a *c.* 1:50 dilution in synthetic medium of an overnight (18–19½ hr) culture of the test organism was placed, immediately after diluting, on the upper surface of the prepared culture chamber membrane, under which the medium was already circulating, and covered with a well polished cover glass (no. 1, 7/8 in.), care being taken to exclude air bubbles. A drop of immersion oil was then placed on the cover glass and the membrane brought into focus. After allowing about 15–30 min for the organisms to settle, a field of 7–15 well spaced organisms was selected.

The entire preparation of the inoculum and the subsequent observations were carried out in a room controlled at 37°.

Recording. All generation times were measured by continuous observation to ±1 min, from and to the moment of appearance of light between mother and daughter cells; this corresponds roughly to Powell's cell wall generation time τ, (Powell, 1958). As the moments of inception of the original cells of the inoculum were not known no generation time could be ascribed to them. The time between inoculation and the first division was therefore recorded as the 1st division time.

Powell (1958) wrote: "An organism was considered non-viable (i) if it became dimly visible under phase contrast and then showed no sign of growth within about 1 hr, (ii) failing (i), if it showed no sign of growth during about 3 hr." These criteria were adopted here, and in addition long forms were considered non-viable if they failed to divide within about 2 hr and showed no sign of growth within about 1 hr subsequently.

RESULTS

Individual variability. The cells of the inoculum, which varied in size, behaved in one of a number of ways. Usually the largest divided first, but this was not always the case. Smaller cells usually enlarged before division, but sometimes divided without appreciable enlargement to yield coccal forms, usually of reduced viability. By plotting the number of cells in a single clone (a group of cells derived from one individual) against time, clone histories were obtained (Fig. 1). By recording on the same time axis the clone histories of the cells initially present in one field of observation, the clonal growth of a population (population history) was obtained. Fig. 2 shows the clone and population histories of a field which initially had 14 cells. Although the divisions in individual clones showed synchronization, in the population as a whole divisions were distributed more or less evenly over the entire period of growth (Fig. 3), so that some clones had completed all the divisions leading to, say, the 3rd generation before others had yielded any 3rd generation individuals at all.

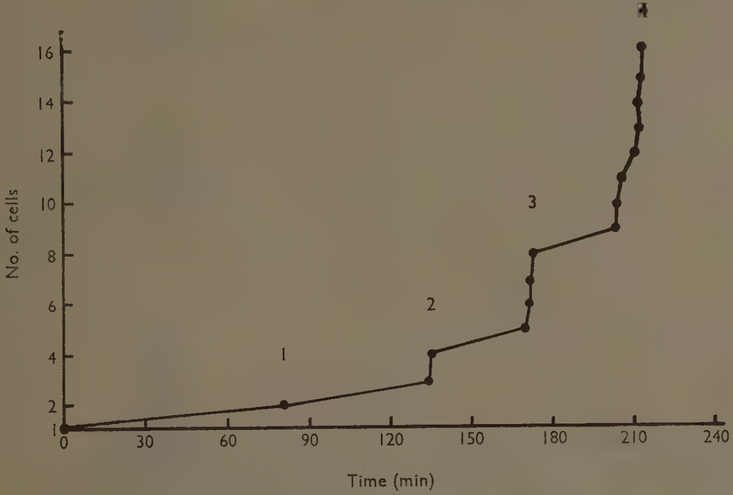

Fig. 1. The history of a single clone of *Escherichia coli*. All the daughter cells for four generations have survived to yield a population of 16 cells in 215 min after the moment of inoculation, at time 0.

Population development. The chronological development of a population, disregarding clonal history, was obtained by plotting each division at the time of its occurrence. One such, comprising the 14 clonal histories of Fig. 2, is shown in Fig. 3. Each generation lasted about 1 hr, but overlapping occurred in later generations. The curve shows the increasingly fast production of increasingly large numbers of organisms, i.e. a gradually increasing growth rate $\left(\frac{\mathrm{d}n}{\mathrm{d}t}\right)$ and specific growth rate $\left(\frac{1}{n}.\frac{\mathrm{d}n}{\mathrm{d}t}\right)$, where n is the number of cells and t the time.

Histograms of the frequency distributions of the generation times for the formation of the 2nd, 3rd and 4th generations, using the combined data for all experiments, are shown in Fig. 4. A gradually decreasing scatter is indicated by the decreasing inter-quartile distances (Table 1). The frequency distribution of all the generation times showed a marked positive skewness.

The mean generation time for the 1626 divisions observed was 52·5 min, and 81% of all the generation times fell within ±15 min of the mean. Of all the generation times, the longest in which two viable daughter cells were formed was 92 min; it occurred in the formation of the 2nd generation. The shortest was 20 min, and occurred once in the formation of organisms both of the 2nd and 4th generations. The longest time for a 1st division which yielded two viable daughter cells was 176 min, and the shortest was 30 min.

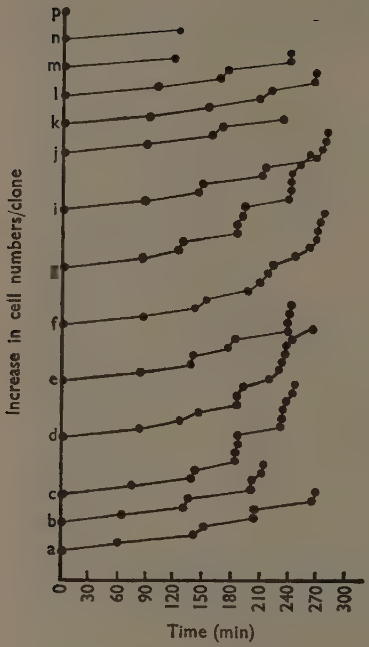

Fig. 2. A population history composed of 14 clonal histories, a–p, each letter marking a single cell of the inoculum and each full circle denoting one cell division. The clones developed at different rates, and the earliest and latest dividers showed reduced viability. Cell p was non-viable.

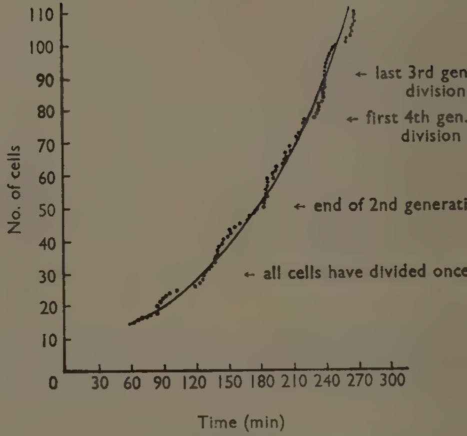

Fig. 3. The chronological development of a population from 14 individual cells during 4½ hr after inoculation. Every cell division is recorded and each point denotes the addition of one organism to the total. The data are the same as in Fig. 2.

Table 1. *The parameters of the frequency distributions of the generation times in three successive generations of* E. coli *growing in liquid synthetic medium*

Parameter	Value or range (in min) for generation		
	2	3	4
Mean	56	55	49
Mode(s)	40–45 50–55	55–60	40–45
Median	54	55	48
Quartiles	45, 66	46, 63	42, 55
Interquartile distance	21	17	13

Viability. Of the 154 individuals in the various inocula, 16 did not divide during the time of observation, an initial non-viability of 10·4%, whereas among the 1626

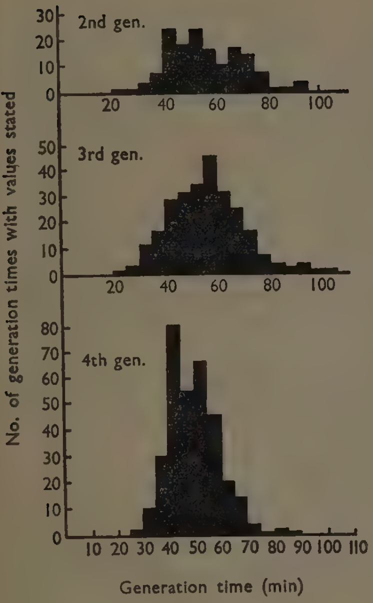

Fig. 4. Histograms showing the frequency distributions of generation times for the 2nd, 3rd and 4th generations in all experiments. The numerical values are shown in Table 1.

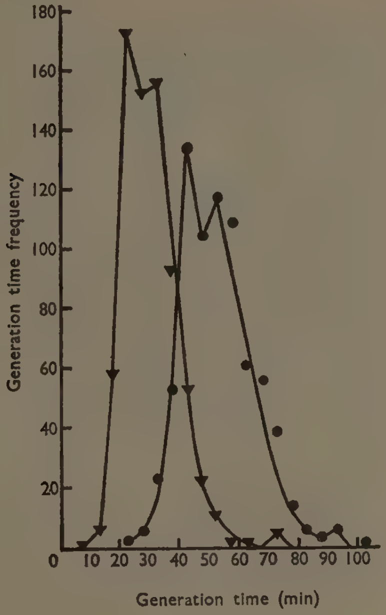

Fig. 5. The similarity between the frequency distribution of the generation times obtained from the combined data for the 2nd, 3rd and 4th generations in the present work with *Escherichia coli* in liquid synthetic medium (circles) and that derived from the data of Kelly & Rahn (1932) for *Bacterium aerogenes* on agar (triangles).

cells formed during the observations only 94 were non-viable, a lag phase non-viability of 6%.

Alternation of generations. In any clone history a generation of long mean generation time was often followed by one of short mean generation time, or vice versa; this was so in 52% of the 65 cases. The amplitude of this alternation decreased with increasing age of the clone.

Because of the number of cells present after the 4th generation later divisions could not easily be recorded and results were obtained for only a few 5th generations. These, so far, suggest further decreases in the generation times.

Cell size. No systematic records of cell size were made, but considerable (and apparently random) differences were observed, both at the time of inoculation and subsequently. Occasionally, individuals failed to divide but continued to grow, resulting in long forms.

DISCUSSION

After inoculation the cells did one of three things, and could be grouped accordingly. Group (a) comprised cells showing an indentation, the first visible sign of division. These continued to divide and gave the shortest 1st division times observed; but the fission process was not completed for at least 30 min. Group (b) comprised most of the other cells which, irrespective of their size, produced daughter cells after widely differing but comparatively long 1st division times. Group (c) consisted of the few cells which failed to divide.

Of the cells that divided, those with the shortest and those with the longest 1st division times, groups (a) and late (b), produced the highest proportion of non-viable daughter cells. Viable daughters of group (a) cells tended to have 2nd generation times considerably longer than the mean for that generation. It would seem that on transfer to a new environment, a cell may be either more or less susceptible to the shock of transfer, according to its physiological age.

Kelly & Rahn (1932), using the technique of Orskov (1922), observed the development at 30° of clones from single cells of *Bacterium aerogenes* and *Bacillus cereus* inoculated from young liquid cultures on to agar. They did not consider the lag phase to extend beyond the 1st recorded generation, but calculation from their figures (for one experiment reported) gives mean generation times of 23·6, 23·4 and 22·2 min for the 2nd, 3rd and 4th generations respectively, which we believe to be in accordance with the present findings (Table 1). Moreover, a frequency distribution curve of generation times constructed from the combined results of Kelly & Rahn (1932) is remarkably similar to that obtained from the present results with *E. coli* (Fig. 5), and it seems possible that the apparent multimodality may be a genuine characteristic of growth in the lag phase.

Wilson (1922) compared plate counts with total counts obtained with a counting chamber, and concluded that ". . . even during the log phase the percentage of viable organisms seldom rises above 90% of the total." Régnier, David & Kaplan (1932) and Jordan & Jacobs (1944), and Wilson (1926) who used agar cultures, came to a similar conclusion. Valentine & Bradfield (1954) estimated by their urea method the proportion of non-viable log phase cells at less than 2%, while Powell (1958) considered it to be less than 1%. In contrast to the present findings, Kelly & Rahn (1932) found no instance of lag phase 'mortality'; but the high degree of non-viability observed in this investigation cannot alone account for the lag phase.

The alternation of short and long generations which was noted can probably be accounted for by considering the various possible arrangements of consecutive mean generation times. These are: long to short to short, short to long to long, short to long to short, and long to short to long. Thus 50% of the sequences would be expected to show alternation, and the observed value of 52% accords well with this. There was a curious difference in the frequency of the two types of alternation, in that in 38% of the sequences the alternation was from short to long to short, while in only 14% was it from long to short to long in the 2nd, 3rd and 4th generations respectively.

In most cases the 1st division times were long compared with the subsequent generation times, yet the technique was such that the possibility of thermal shock was excluded. This, taken together with the wide scatter of the 1st division and 2nd generation times, suggests that the fresh medium was exerting a bacteriofrenic action. With the system used it is difficult to believe that the accumulation of extracellular metabolites discussed by Hinshelwood (1946) and Wilson & Miles (1955) as important for the initiation of growth could have occurred: but their absence may have contributed to delayed division.

The present observations suggest that the lag phase which is normally observed in stationary cultures is due to and affected by four factors: (1) a period (minimum *c*. 30 min) in which no cell divisions occur, (2) a further period (up to 3 hr) before all the organisms of group (b) have divided, (3) the presence of comparatively large numbers of non-viable organisms as compared with the log phase, and (4) a prolongation of generation times into the 3rd, 4th and possibly the 5th generations.

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A COMBINED TEST FOR PENICILLINASE PRODUCTION AND PENICILLIN SENSITIVITY OF STAPHYLOCOCCI

By A. G. INNES

Co-operative Wholesale Society Ltd., Technical Research Department, Manchester

SUMMARY: A method described for the demonstration of penicillinase production by staphylococci, a modification of that described by Voureka & Hughes (1949), is less laborious than the original and gives results which are readily interpreted.

All naturally occurring penicillin resistant staphylococci are producers of penicillinase. For most practical purposes the ability to produce penicillinase can be inferred from the result of a sensitivity test carried out by one of the conventional methods. However, penicillin destroying staphylococci are susceptible to the killing effect of penicillin, and for this reason an erroneous impression of sensitivity to penicillin can be obtained from both tube dilution and plate diffusion tests if too small an inoculum is used (Spink, Ferris & Vivino, 1944). In tests with a sufficiently large inoculum most strains are either clearly sensitive or resistant, but an occasional very weak producer of penicillinase may give equivocal results, such as a small zone of inhibition in a disc sensitivity test. When this happens, a sensitive and easily performed test for penicillinase production is valuable.

The following method, adapted from that of Voureka & Hughes (1949), has proved useful in our hands.

Method

Two ml of an overnight broth culture of the standard Oxford staphylococcus were added to 100 ml of molten nutrient agar at 50°, mixed well and poured into plates to a depth of 2–2.5 mm. When set, a ditch was cut in the agar using a sterile scalpel and filled with nutrient agar containing 10 i.u. of penicillin/ml. Prepared plates were well dried before use. Streaks of the staphylococci to be tested, from overnight or preferably 6 hr broth cultures, were made up to and across the ditch. Five streaks could be conveniently planted on one plate. Inoculated plates were incubated at 37° and results were read after 18 hr. Any fully sensitive staphylococcus can be used in place of the standard Oxford staphylococcus.

Results

After incubation the Oxford staphylococcus was inhibited in the region of the ditch and the edge of the zone of growth was parallel with the ditch. Whenever the test staphyloccus produced penicillinase there was an arrow-shaped encroachment of the growth of the Oxford staphylococcus towards the ditch along the streak of the test

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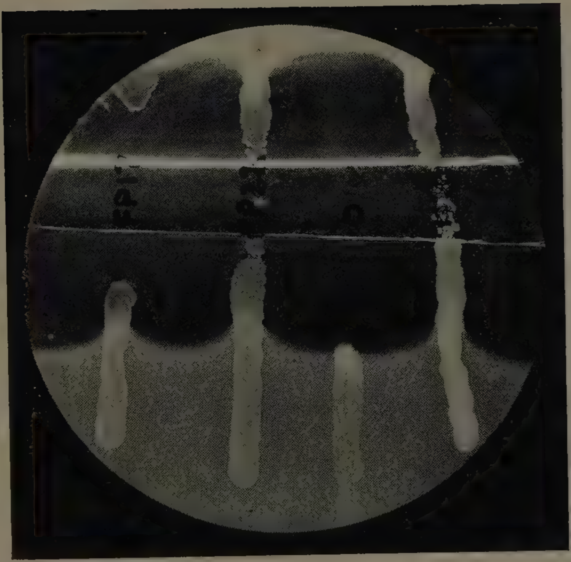

A. G. INNES — A COMBINED TEST FOR PENICILLINASE PRODUCTION AND PENICILLIN SENSITIVITY OF STAPHYLOCOCCI. PLATE 1

organism, as shown in Plate 1. Penicillinase production by the organisms was thus readily demonstrated and the method proved to be particularly sensitive in detecting the production of small quantities of penicillinase which might have been missed had conventional methods been used.

The author wishes to thank Mr. A. Glover for granting permission and facilities for the investigation to be carried out. This paper is published with the permission of the Directors of the Co-operative Wholesale Society Limited, Manchester.

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EXPLANATION OF PLATE

Ditch plate, showing encroachment of growth of the Oxford staphylococcus towards the ditch along the streaks of penicillinase producing staphylococci.

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TELLURITE-EGG AGAR, A SELECTIVE AND DIFFERENTIAL MEDIUM FOR THE ISOLATION OF COAGULASE POSITIVE STAPHYLOCOCCI

BY A. G. INNES

Co-operative Wholesale Society Ltd., Technical Research Department, Manchester

SUMMARY: The preparation and use of tellurite-egg agar (TEA), a medium for the isolation of coagulase positive staphylococci, are described. The medium, based on that of Ludlam (1949), was non-inhibitory to coagulase positive staphylococci and colonies on it of these organisms could easily be distinguished from contaminating types. Comparisons were made between the medium and three other media in common use for the isolation of coagulase positive staphylococci.

FOR THE ISOLATION of coagulase positive staphylococci from materials which are liable to be contaminated with many other organisms it is desirable to use a medium which will produce distinctive colonies of staphylococci while suppressing the growth of contaminating organisms. A number of media have been designed for this purpose. Ludlam (1949) described a selective medium for the isolation of coagulase positive staphylococci from heavily contaminated material, and it has been used for several years in this laboratory for the isolation of staphylococci from foodstuffs. When first tested, however, it proved inhibitory to certain strains. Reduction of the tellurite concentration to 1:32,000 rendered the medium less inhibitory but allowed the growth of aerobic sporeformers and micrococci. Other media tested from time to time were those of Chapman (1945, 1946), Barber & Kuper (1951) and Zebovitz, Evans & Niven (1955); but for our purpose these proved inferior to Ludlam's medium. Occasionally, however, this medium gave disappointing results in the examination of heavily contaminated foodstuffs, due to the overgrowth of the staphylococci by sporing organisms. It was also noted that certain strains of coagulase positive staphylococci did not always give the typical colonial appearance on the medium. Recently Klemperer & Haughton (1957) described a medium containing fibrinogen and penicillin for the isolation of penicillin resistant coagulase positive staphylococci; but the coagulase positive staphylococci isolated from foodstuffs were by no means all penicillin resistant and without penicillin added the medium was not selective.

While examining staphylococci isolated from various sources it was noted that all of 34 enterotoxic strains were egg yolk positive. Gillespie & Alder (1952) found that about 80% of coagulase positive strains of staphylococci isolated from human sources outside hospital were egg yolk positive, whereas all of the 60 coagulase negative staphylococci they examined were egg yolk negative. Alder, Gillespie & Herdan (1953) found that the coagulase negative staphylococci of human origin were all egg yolk negative and that most of the coagulase positive staphylococci of human origin were egg yolk positive.

In view of these findings it was decided to investigate the possibility of utilizing the egg yolk reaction in the preparation of a solid selective and differential medium for coagulase positive staphylococci. No coagulase negative staphylococci would give a zone of opacity on such a medium and if the medium were based on Ludlam's original formula all coagulase positive staphylococci should give distinctive colonies irrespective of their egg yolk reaction.

METHODS

Preliminary experiments. A medium was prepared by adding an equal quantity of egg yolk broth (Oxoid) to double strength Ludlam's agar base, potassium tellurite being added to give a final concentration of 1:32,000. On this medium egg yolk positive coagulase positive staphylococci formed dark grey colonies surrounded by opaque zones after incubation at 37° for 30–48 hr. Inhibition of certain strains occurred, however, and sporing organisms were not inhibited to any great extent. When the medium was filtered before the addition of the egg yolk broth it tended to be even more inhibitory to staphylococci. Since the inhibitory agent was evidently the potassium tellurite it was decided to reduce the concentration further to 1:40,000. With a low tellurite content many contaminating organisms would be able to grow on the medium, and to counteract that the Lab-Lemco content of the medium was reduced to 0·3%. The lithium chloride content was not altered because it was originally included to inhibit enterococci, colonies of which might be similar to those of coagulase positive staphylococci. In order to reduce the opacity of the medium, which was brought about when the egg yolk broth was added, 1% of sodium chloride was incorporated. The medium then gave satisfactory results. There was no evident inhibition of coagulase positive staphylococci, colonies of which were easily distinguished from those of the coagulase negative strains which grew. There was also greater inhibition of sporing organisms. Later, concentrated egg yolk emulsion (Oxoid) was substituted for the egg yolk broth to avoid the need to prepare double strength basal medium.

Final medium. The final formula for the medium was therefore as follows: Mannitol, 10 g; K_2HPO_4, 5 g; NaCl, 10 g; LiCl, 5 g; peptone (Bacto or Evans), 10 g; Lab-Lemco, 3 g; distilled water, 900 ml. The ingredients were dissolved by heating, the pH adjusted to 9·2 with thymol blue or electrometrically and 20 g of agar added and dissolved by heating. The medium was then distributed in 90 ml quantities and autoclaved at 10 lb pressure for 20 min. Before use, 10 ml of concentrated egg yolk emulsion (Oxoid) prewarmed to 50° and 1 ml of a sterile aqueous 1:400 solution of potassium tellurite were added to 90 ml of molten base at 50°, mixed well avoiding air bubble formation, and plates poured. The surface of the medium should be well dried before use. The final pH of the medium is 8·0–8·2.

RESULTS

Comparison of tellurite-egg agar with other media. Experiments were carried out with various strains of coagulase positive staphylococci to compare the tellurite-egg agar (TEA) with other media. The numbers present in dilutions of broth cultures of these organisms were estimated by the surface viable drop technique of Miles & Misra (1938). Bacto blood agar base was used as a control medium.

The results of an experiment in which TEA was compared with Ludlam's medium (LUD) are given in Table 1. The minimum difference between the mean numbers of colonies on the media required for significance at the 5% level was $\pm 10{\cdot}3$, so that whereas the mean number of colonies on the TEA was not significantly different from that on the control medium ($P>0{\cdot}05$), the difference between the mean numbers on LUD and the control was highly significant ($P<0{\cdot}001$).

Table 1. *A comparison of the growth of a selected group of coagulase positive staphylococci on tellurite-egg agar (TEA) and Ludlam's medium (LUD)*

Strain no.	Mean colony count* (cells/0·02 ml) on		
	Control†	TEA	LUD
1	80·0	86·6	72·8
8	157·8	159·6	126·6
9	72·0	61·0	56·2
47	64·2	70·0	48·6
147	51·8	39·8	21·0
161	51·8	72·6	50·2
Means	79·6	81·6	62·6

* From 10^{-5} dilution. † Bacto blood agar base.

Table 2 shows the results of a similar experiment in which the control medium, TEA, the tellurite glycine agar (TG) of Zebovitz *et al.* (1955) and staphylococcus medium no. 110 (Chapman, 1946) were compared. The minimum difference between the mean numbers of colonies on the media required for significance at the 5% level

Table 2. *A comparison of the growth of a selected group of coagulase positive staphylococci on tellurite-egg agar (TEA), tellurite glycine agar (TG) and staphylococcus medium no.* 110 (110)

Strain no.	Colony count* (cells/0·06 ml) on			
	Control†	TEA	TG	110
8907	98	111	84	73
697	104	119	0	104
1074	92	107	102	109
2023	160	163	162	186
2072	56	51	53	56
2110	37	38	36	44
2204	82	96	76	83
1970	113	96	103	100
1971/1	82	109	96	81
1971/2	186	166	150	160
1	197	212	146	184
8	111	114	79	95
Means	36·6	38·4	30·2	35·4

* Sum of 3 replicate counts from 10^{-5} dilution. † Bacto blood agar base.

was ±4·5. Both TEA and staphylococcus medium no. 110 gave counts which were not significantly different from those given by the control medium ($P > 0{\cdot}05$) but the difference between the counts on the tellurite glycine agar and on the control medium was highly significant ($0{\cdot}001 < P < 0{\cdot}005$). There was no evidence that TEA inhibited staphylococci, and in fact it gave a slightly better yield than the control medium.

Coagulase positive staphylococci on TEA. The colonies produced by egg yolk positive coagulase positive staphylococci on TEA after incubation at 37° for 24 hr were dark grey with a narrow pale periphery, smooth, glistening, low-convex, entire and 1–2 mm in diameter. They were surrounded by clear or opaque zones 2·5–4 mm in diameter. After incubation for 30–48 hr the clear zones became opaque. The rate at which the opacity developed varied with the strain of staphylococcus. Occasionally the clear zones were replaced by surface zones having an oily appearance which were easily detected when the plates were examined by reflected light. Egg yolk negative coagulase positive staphylococci produced colonies which were similar in all respects to those of the egg yolk positive strains except that they were never surrounded by opaque zones, though sometimes by clear zones.

Although most strains of coagulase positive staphylococci gave the typical colonial appearance on the medium after incubation at 37° for 24 hr it was necessary to incubate inoculated plates for 48 hr to ensure that all such organisms were detected. One strain of staphylococcus isolated from a foodstuff gave pale colonies after incubation for 24 hr but gave typical dark grey colonies after 48 hr. The organism was coagulase negative when tested by the slide method of Cadness-Graves *et al.* (1943). When 2 drops of an overnight culture in broth were added to a tube containing 0·5 ml of rabbit plasma which had been diluted 1:10 with sterile saline a clot was obtained after incubation at 37° for 3 hr, thus showing a deficiency of bound coagulase in the organism (Duthie, 1954). After repeated subcultures had been made the organism became weakly coagulase positive when tested by the slide method. A subculture of the organism on to Ludlam's medium gave a restricted growth of small pale colonies after incubation at 37° for 48 hr. Normally, colonies having that appearance would be disregarded.

The clear zones produced in TEA by coagulase positive staphylococci were due to the action of the end products of mannitol fermentation by the organisms on the precipitate of phosphates in the medium; thus these zones served as a useful indicator of mannitol fermentation by the organisms.

Trials were carried out by adding small numbers of enterotoxic staphylococci to liquid whole egg. These were either plated directly on to TEA or enriched in salt meat broth (Oxoid) by incubation at 37° for 24 hr before plating out. All the staphylococci were recovered, practically pure growths being obtained in all cases and the colonies had the typical appearance reported above.

Coagulase negative staphylococci on TEA. These were greatly inhibited and when they did grow they formed small pale colonies from which the colonies of the coagulase positive staphylococci were easily distinguished. Egg yolk positive sporeforming aerobes produced opaque zones on the medium without tellurite, but when potassium tellurite was added they were either inhibited or produced brownish colonies not surrounded by opaque zones. Coagulase positive staphylococci were thus easily distinguished from *Bacillus cereus*, the only other egg yolk positive organism liable to be isolated with any frequency from foodstuffs.

Pigment production on TEA. The medium without the potassium tellurite was excellent for pigment production. The amount of pigment produced by coagulase positive staphylococci after incubation on the medium at 37° for 24 hr was somewhat greater than that produced on staphylococcus medium no. 110, which was reported by Evans (1948) to be superior to infusion agar in the production of pigment by coagulase positive staphylococci.

DISCUSSION

Coagulase positive staphylococci may be present in infected materials in small numbers only, and this makes their isolation often tedious and difficult. Media in current use for the isolation of staphylococci were found to be partially, and in some cases totally, inhibitory to certain strains of staphylococci and it was by no means certain, therefore, that in all cases a total recovery of the organisms was obtained when these media were used. The tellurite-egg agar designed for the isolation of coagulase positive staphylococci from material in which few of the organisms were liable to be present was found to be efficient in this respect. It has been used in this laboratory for some time for the examination of foodstuffs suspected of containing enterotoxic staphylococci, and there has been no indication that it failed to detect the coagulase positive staphylococci present. No significant inhibition of any strain of coagulase positive staphylococcus yet encountered occurred on the medium. Moreover, the ease with which egg yolk positive staphylococci were detected on the medium in the presence of contaminating organisms made it superior to other media in current use. Since the enterotoxic staphylococci tested during the investigation were found to be egg yolk positive it was considered that the medium would prove to be a useful aid in the diagnosis of staphylococcal food poisoning.

The author wishes to thank Mr. A. Glover for granting permission and facilities to carry out the investigation. This paper is published with the permission of the Directors of the Co-operative Wholesale Society Limited, Manchester.

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ANTIBIOTIC SUBSTANCES FROM A STRAIN OF *BACILLUS SUBTILIS*

By M. PURKAYASTHA and P. NANDI

Department of Microbiology, The Bose Institute, Calcutta, India

SUMMARY: Strain B_7 of *B. subtilis*, isolated from soil collected from the gardens of the Bose Institute, Calcutta, was found to elaborate at least four different antibiotic substances. Fraction I was a brick red amorphous powder with certain resemblances to the bacillomycin group of antibiotics, soluble in alcohols, pyridine, etc., but insoluble in ether, water and acids. It had an ultraviolet absorption maximum at 276 mμ, melted with decomposition at 311°, and was a polypeptide positive to ninhydrin. On hydrolysis 12 amino acids were found. Fraction II was a white amorphous powder soluble in alcohols and ether but insoluble in water and petroleum ether. It melted at 158–162° and was a ninhydrin positive polypeptide composed of 7 amino acids. Fraction III was a yellow amorphous powder soluble in water and acetone, less soluble in ether and practically insoluble in chloroform. It was not a polypeptide. Fraction IV was a yellowish white amorphous powder with solubility characteristics similar to those of fractions I and II. It was a polypeptide composed of 10 amino acids.

Many antibiotic producing bacteria were isolated during an extensive survey of antibiotic producing bacteria from Indian soils (Purkayastha & Nandi, 1960). Most of them belonged to the genus *Bacillus*, and *B. subtilis* comprised the largest group. One of the *B. subtilis* strains, designated B_7, which possessed strong antifungal but weak antibacterial properties has been investigated in some detail and in this paper an account is given of the isolation and purification of the active substances produced.

Methods

Organism used. This was a culture isolated from a crowded plate of a soil sample collected from the garden of the Bose Institute, Calcutta, and designated B_7. Its identity as a strain of *B. subtilis* had already been established by Purkayastha & Nandi (1960) by the usual morphological and biochemical tests.

Antibiotic production. For this the organism was grown at 30° for 48 hr in 500 ml Ehrlenmeyer flasks each containing 150 ml of sucrose-yeast extract broth. Shaking was particularly useful for increasing growth in these relatively deep cultures. The optimum temperature for antibiotic production was 30–34° and the optimum pH was 6·8. Production reached its maximum after 48 hr.

Separation of the active substances. Three methods were used: solvent extraction, adsorption on alumina, and adsorption on ion exchange resins.

The alumina (Brookmann's) was washed thoroughly with water or dilute acetic acid and alcohol before use. The ion exchange resins tried were: Amberlite IR-4B, IR-120, IRA-400 and IRC-50; Dowex-1, Dowex-2 and Dowex-50. They were freed from fine particles by repeated washing and decantation and the soluble impurities were removed by several cycles of alternate treatment with N NH_4OH and N HCl.

The alumina columns were 20 cm long and 1 cm in diameter, and the resin columns were 33 cm long and 1 cm in diameter.

After adsorption, the antibiotic substances were eluted with suitable agents and the fractions collected in a Shandon Automatic Fraction Collector, which enabled the active principles to be located precisely.

Purity, estimation and nature of the active substances. The homogeneity of the active principles was studied by both one- and two-dimensional paper chromatography on Whatman's no. 1 filter paper, using various solvents, and by paper ionophoresis.

The constituents were estimated colorimetrically where possible, using a Klett-Summerson Photoelectric Colorimeter with filter no. 42, or by bioassay, by the agar cup plate method.

The ultraviolet absorption spectra of the substances isolated were determined with a model DU Beckman Spectrophotometer, using 90% ethanolic solutions of the compounds.

The active substances were hydrolyzed with 6N HCl for 48 hr at 110° in sealed hard glass tubes. After complete evaporation of the HCl, the residues were subjected to paper chromatography for separation and identification of the amino acids present. Comparison of the Rf values, and co-chromatography with authentic samples, were used in the identifications.

RESULTS

Fractions obtained by solvent extraction

Several litres of the culture fluid were centrifuged in a Sharples centrifuge to remove the bacterial cells. The fluid was acidified with dilute HCl and the precipitate which formed was centrifuged down and washed with distilled water. It was then treated with *n*-butanol, and part of it went into solution. The butanol soluble fraction was antimicrobially active. The yield of this partially purified fraction was approximately 40 mg/l. of culture fluid. It was redissolved in a small volume of 90% methanol, an equal volume of a 1:1 mixture of petroleum ether (40–60°) and benzene was added and the whole was shaken in a separating funnel.

After separation, the brick red lower (methanolic) layer was withdrawn. It was shaken several times with further quantities of the petroleum ether-benzene mixture, and the final methanolic layer was evaporated to dryness and then dissolved in 95% ethanol. To this diethyl ether was added, when a flocculent precipitate formed. This was taken up in 95% ethanol and reprecipitated several times, and finally the residue was washed successively with large volumes of water, petroleum ether and ether. The residue was finally dissolved in dry methanol again and filtered. On evaporation to dryness this filtrate gave a brick red mass which was labelled fraction I.

The upper petroleum ether-benzene layer plus the combined petroleum ether-benzene washings from the treatment of the lower layer were then washed several times with methanol and evaporated to dryness. The residue was dissolved in ether, filtered and the ethereal solution evaporated to dryness. The residue was then dissolved in ethanol and a few drops of water were added, when precipitation took place. The precipitate was washed with water, dissolved in ether and the solution filtered. On evaporation a white powder was obtained, which was labelled fraction II.

Fractions obtained by adsorption chromatography

Adsorption on alumina. The precipitate obtained by acidifying centrifuged culture fluid with HCl was dissolved in a small volume of ethanol. The solution was filtered, and the filtrate was allowed to percolate slowly through an alumina column. No antibiotic activity was detected in the effluent. Most of the active material was adsorbed near the top of the column. The best method of eluting the antibiotics was to use *n*-butanol adjusted to an acid reaction.

Aliquots of each 5 ml fraction collected were bioassayed. Material corresponding to fraction II (see below) was largely eluted in tube no. 3, and material with the properties of fraction I in tubes 4, 5 and 6. A yellow substance distinct from fractions I and II and designated fraction III was obtained from tubes 12–14. This was not obtained by solvent extraction.

Adsorption on resins. Of the various resins tried IRA-120 proved best. It was used in the H^+ form, obtained by treatment with N HCl followed by washing with distilled water to remove the excess of acid. A solution in N NH_4OH of the acid precipitate from the clarified culture fluid was allowed to pass at the rate of 1·0 ml/3 min through a column of the resin. The effluent had no antibiotic activity.

Most of the active material was adsorbed near the top of the column. The resin column was then washed with 50 ml of water and 36 ml of 0·1 N acetic acid passed through, followed by 176 ml of HCl of increasing strength (1–6N). Finally, 150 ml of 6N HCl in methanol was passed through. The material in each 5 ml fraction was estimated colorimetrically and by bioassay. The first material to be eluted from the resin was different from anything obtained by the other methods and was designated fraction IV. The substance called fraction III was largely eluted with 2–4N HCl and that called fraction I with the 6N HCl in methanol. Material resembling fraction II, which in solution was colourless and could not therefore be detected colorimetrically, was obtained as a white mass when the contents of tubes 93–127 were evaporated to dryness.

Homogeneity, properties and activities of fractions I-IV

The homogeneity of the fractions obtained by solvent extraction and from the eluates of the alumina and resin columns was proved by paper chromatography and by paper ionophoresis. Their Rf values in a variety of solvent systems are given in Table 1.

Fraction I. This was a brick red amorphous powder soluble in methanol, ethanol, *n*-butanol, *n*-amyl alcohol, pyridine and acetone but insoluble in ether, petroleum ether, benzene, water and acids. It was soluble in alkali, including 5% $NaHCO_3$ solution. It melted with decomposition at 311°. Its ultraviolet absorption spectrum showed a maximum at 276 mμ. It was fairly stable in aqueous solution between pH 6·0 and 8·0. No diminution in potency was noticed after heating at 90° for 30 min, and after 6 hr destruction was only partial, but it was complete after heating for 30 min at 120°. It was affected only slightly, if at all, by pepsin, trypsin or papain at different pH values. It was not inactivated by cysteine HCl and was precipitated by protein precipitating agents, e.g. trichloracetic acid, tannic acid and lead acetate. It gave a positive test with ninhydrin and negative tests with Fehling's solution and $FeCl_3$. It proved to be a non-dialyzable polypeptide. After hydrolysis 12 amino acids were detected, namely aspartic acid, serine, glutamic acid, threonine, alanine, tyrosine, proline, valine, leucine, arginine, lysine, and glycine.

Table 1. *The Rf values in different solvent systems of the four antibiotics of* B. subtilis *strain* B_7

Solvent systems	Rf values of fractions			
	I	II	III	IV
Water	0·08	0·08	0·81	0·79
Ethanol, 20%	0·08	0·08	0·67	0·71
Ethanol, 50%	0·12	0·13	0·09	0·73
Ethanol, 80%	0·15	0·14	0·09	0·72
Ether-alcohol-water (2:1:1)	0·32	0·32	0·26	0·35
n-Butanol-acetic acid-water (4:1:1)	Solvent front	0·40	0·23	Solvent front

The organisms which were inhibited by fraction I included *Staphylococcus aureus*, *Xanthomonas malvacearum*, *Micrococcus flavus*, *Azotobacter chroococcum*, *Az. indicum*, *Az. vinelandii*, *Streptomyces noursei*, a *Curvularia* sp., *Fusarium vasinfectum*, *Helminthosporium sativum*, *Alternaria solani*, *Trichophyton rubrum* and *Fusarium oxysporum* var. *pisi*. The minimum concentration required for the inhibition of *Helminthosporium sativum* was 2·5 μg/ml.

Fraction II. This was a yellowish-white amorphous powder, soluble in methanol, ethanol, pyridine, ether, benzene and alkalis, including 5% $NaHCO_3$ solution. It was insoluble in water and petroleum ether, but soluble in a mixture of petroleum ether and benzene. It melted at 158–162°. It was negative to Fehling's solution and $FeCl_3$ but ninhydrin positive and after hydrolysis the amino acids aspartic acid, glutamic acid, alanine, tyrosine, valine, phenylalanine and glycine were detected.

In its antimicrobial activity it was very specific, being inhibitory to *H. sativum* and a *Curvularia* sp. only. The minimum concentration required to inhibit *H. sativum* was 8 μg/ml.

Fraction III. This was a yellow amorphous substance soluble in water and acetone, less soluble in ether and almost insoluble in chloroform. It was not a polypeptide. It was active against *H. sativum*.

Fraction IV. This was a yellowish-white amorphous powder soluble in lower alcohols and insoluble in water and acids. After hydrolysis the amino acids glutamic acid, threonine, alanine, tyrosine, proline, valine, leucine, arginine, glycine and aspartic acid were detected. It was active against *H. sativum* only.

DISCUSSION

Strain B_7 is an antifungal strain with weak antibacterial activity, whose antibiotic powers are largely due to the brick red amorphous powder which has been called fraction I. This resembles the bacillomycin group of antibiotics (Sharon *et al.* 1954) and mycosubtilin and fungistatin, which have also been isolated from *B. subtilis* cultures and which show marked antifungal activity, in being an extracellular, acidic, non-dialyzable heat stable polypeptide not affected by papain or trypsin and soluble in non-polar organic solvents. However, it differs from these in its antimicrobial spectrum and in certain physical and chemical properties, including its amino acid composition. The difference in antimicrobial spectrum lies in its inactivity towards *Aspergillus niger*, including eight mutant strains developed by ultraviolet irradiation, whereas almost all the members of the bacillomycin group are active against *A. niger*. Also, fraction I contains twelve amino acids, aspartic acid, glutamic acid, serine, tyrosine, threonine, valine, leucine, proline, arginine, lysine and glycine and markedly differs in composition from bacillomycin as described by Landy *et al.* (1948), the active substance isolated by Babad *et al.* (1952), the bacillomycin A of Cercos & Castronovo (1951) and bacillomycins B and C (Sharon *et al.* 1954). The antifungal substance isolated from strain B(X) of *B. subtilis* by Nandi & Sen (1953) has an amino acid composition similar to that of bacillomycin A and the material isolated by Babad *et al.* (1952) and called bacillomycin R by Sharon *et al.* (1954), except that an unidentified compound located near phenylalanine on the paper chromatograms was also present. But only a trace of glutamic acid was present in the antibiotic of strain B(X) whereas in fraction I of strain B_7 it was present in large amounts. Further, the antibiotic from strain B(X) strongly inhibits *Penicillium notatum* as well as *A. niger*, unlike fraction I. Fraction L of iturine (Delcambe, 1952*a*,*b*), a polypeptide having antifungal properties, also inhibits *P. notatum*; but its amino acid composition (aspartic acid, glutamic acid, leucine, isoleucine, proline and tyrosine) is very different from that of fraction I. '*Aspergillus* factor' and '*Rhizoctonia* factor' have certain characteristics in common with fraction I, but the former is soluble in water and the latter is insoluble in butanol. Mycosubtilin is intracellular, insoluble in the lower aliphatic alcohols and does not contain glutamic acid and serine. The polypeptide fungistatin (antibiotic XG) has more marked antibacterial activities and also has tryptophane as one of its constituent amino acids.

Fraction II has a very narrow spectrum of activity, being inhibitory only towards *Helminthosporium sativum* and a *Curvularia* sp. among the test organisms used, while fractions III and IV inhibit *H. sativum* only. They do not appear to be similar to any of the antifungal antibiotics reported from *B. subtilis*.

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RECIRCULATION WITH ELECTROSTATIC FILTRATION AS A MEANS OF REDUCING BACTERIAL AIR CONTAMINATION

By L. STEINGOLD and L. ASHWORTH

St. Andrew's Hospital, Bow, London, E.3

SUMMARY: Recirculation through a free-standing electrostatic air filter reduced considerably the bacterial population of the air of a small hospital room, particularly during periods of nursing activity. It is suggested that it may be of real value as a further means of combating hospital cross infection.

DURING ENQUIRIES into the possibility of installing electrostatic air filters in the positive pressure ventilating systems to the operating theatres of this hospital, we were informed of the existence of a small free-standing model. We are now aware of the existence of other similar models, including one noted by Sykes (1958). The claims made for this model were such as to suggest that it might be of value in reducing bacterial air contamination, particularly during periods of activity, in small rooms such as isolation side-wards and surgical dressing rooms. One such was therefore borrowed for testing.

THE APPARATUS

The principle of operation of the electrostatic precipitator is illustrated diagrammatically in Fig. 1. The machine is housed in a sheet metal casing, 3 ft 1 in. high, 1 ft 6 in. wide and 1 ft 3 in. deep, and is easily transportable. Air enters the machine from below and is driven upwards by a fan through a nylon mesh filter which removes coarse dust particles. It then passes a series of wires bearing a 13,000 V electric charge. These ionize the dust in the air, rendering it electropositive. The air then passes between a series of electromagnetic plates charged at 6,500 V, the negative

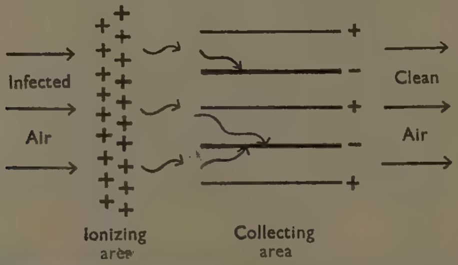

Fig. 1. A diagrammatic illustration of the principle of the electrostatic air filter.

plates of which attract and hold the positively charged dust particles. The dust free air is discharged through mesh grills at the front and sides near the top of the casing. It is claimed that the apparatus removes over 98% by weight of the solid matter in the air, including dust, pollen, bacteria and viruses.

EXPERIMENTS AND RESULTS

Control of airborne organisms during nursing activity. The machine was installed at the foot of the bed in a single bed side-ward of about 1,600 ft³ capacity. A patient suffering from a surgical infection was transferred to this room and observations made on the bacterial contamination of the air of the room by means of a slit sampler. Counts were made throughout a series of working days with the filter in and out of use on alternate days. Typical results are presented in Fig. 2. They show that the bacterial counts were much reduced in the case shown (from an average of 27 to an average of 3/ft³) and in particular the high counts produced during periods of activity were minimized. Similar observations were made, with essentially the same results, in a larger isolation ward of about 2,500 ft³ capacity.

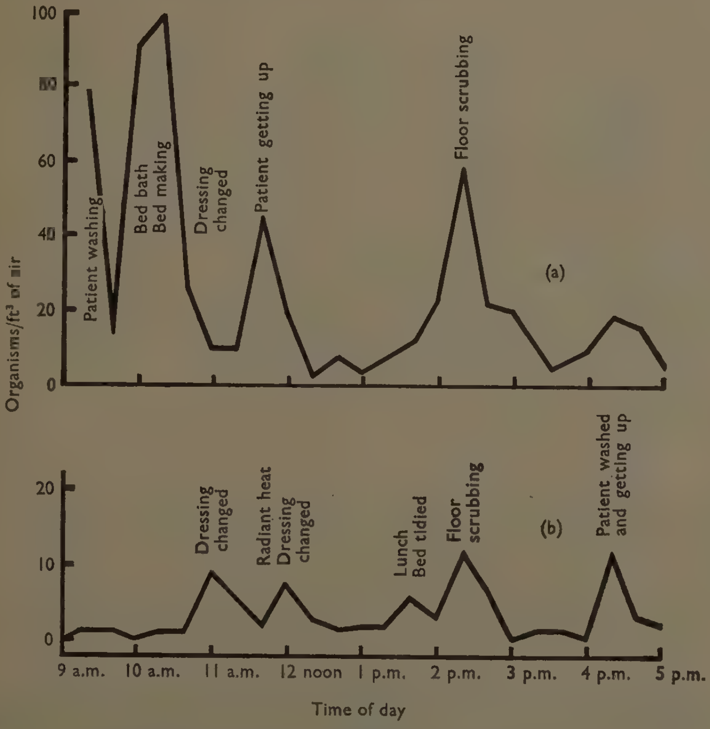

Fig. 2. The effect of electrostatic air filtration on the airborne bacteria in an isolation side-ward: precipitator off (a) and on (b).

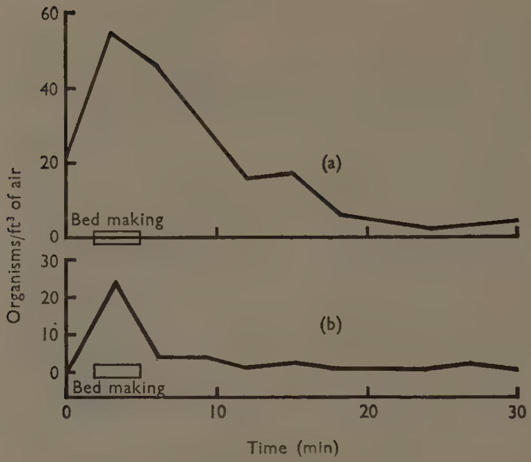

Fig. 3. The reduction by electrostatic air filtration of a peak of bacterial air contamination caused by bedmaking: precipitator off (a) and on (b).

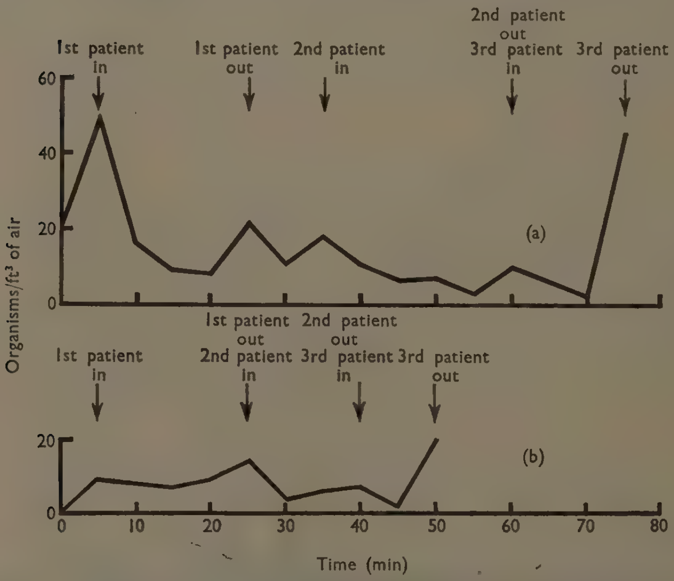

Fig. 4. The effect of electrostatic air filtration on the bacterial contamination of the air resulting from the operation of changing surgical dressings: precipitator off (a) and on (b).

To investigate the significance of the peaks in bacterial air contamination during activity, observations were made before, during and after vigorous bedmaking in a small room. Slit sampler counts were taken every 3 min for a total of 30 min, the bed being made after the first count. As can be seen from Fig. 3, the air filter apparently sterilized the air of the room before the bed was made and considerably reduced the height and duration of the peak of bacterial air contamination resulting from this activity.

Control of airborne organisms during surgical dressing. A small partitioned cubicle of about 2,250 ft^3 capacity was converted to use as a surgical dressing room by installing the electrostatic air filter and putting one of its two beds out of use. On consecutive days three patients (the same three on each occasion) had their dressings changed in this room, the filter being out of use on the first day and in use on the second day for 30 min before and throughout the period of dressing changes. Bacterial counts of the air were taken at intervals of 5 min, with the results shown in Fig. 4. As can be seen, the air filter reduced the initial bacterial count from 21/ft^3 to apparent zero. The average count was also reduced, from 16 to 8/ft^3, and the peak counts of 50 and 46/ft^3 fell to 19 and 14/ft^3. It is noticeable also that at the end of the dressing period the nursing staff tend to relax their precautions and consequently high peaks of bacterial air contamination were produced. It is at this stage, when the dressing room is opened to the main ward, that the risk to the other patients is at its greatest.

Discussion

Hospital cross infection results from an infected hospital environment (Blowers *et al.* 1955; Walter, 1958; Jones, 1960). Lapses in surgical and nursing technique may make it easy for bacteria to enter the body and so establish surgical infections, but hospitals with impeccable techniques are nevertheless plagued by staphylococcal infections, and 'surgical' infections constitute a relatively small proportion of the total cross infection of a hospital.

New patients continually bring bacteria into hospitals, and visitors and the hospital staff add their quota. The bacteria multiply rapidly in infected wounds and sodden dressings as well as in the slightly moist 'dust' which in hospitals must contain dried blood, pus, urine and sputum. They colonize the anterior nares (Gillespie, 1958) and the skin of most, if not all, of the patients and staff. Thus the hospital air is fed, particularly during periods of activity, with large numbers of bacteria from wounds, respiratory passages, blankets, bed curtains, dressings, floors, clothing and so on, and in its movements they become distributed throughout every ward and department.

Positive pressure ventilation reduces the local bacterial air contamination and is widely used in operating theatres; it appears likely that a similar system will be used more widely in surgical dressing rooms (Report, 1959). This system of ventilation is effective because it drives the bacteria out of the room in which they are released. Means of escape to the outer air are provided, but part at least must escape through the doors into the corridors and adjacent wards.

The free-standing electrostatic air filter acts by treating and recirculating the air within a room. It thus minimizes the escape of infected air into hospital corridors or wards because it does not produce any increased air pressure within the room. Under the conditions of test, the particular model examined reduced the average airborne bacterial count throughout a working day to well below 10/ft^3, and during a surgical dressing period, or periods of nursing and cleaning activity, it minimized and shortened the duration of peaks in air contamination.

In use the filter produces some ozone, but this was barely noticeable and did not cause any respiratory or other irritation. The precipitation cell is easily removed from the case for cleaning. Cleaning is normally carried out by hosing the cell, but on one occasion it was washed in a tank of water and the bacterial count of the water taken before and after the treatment. Although the water became very dirty, the results indicated that the liberated dirt was sterile. If this is verified, it would appear that the high voltage ionization of the dust particles, combined with the effect of the ozone, is sufficient to destroy the bacteria in the collected dirt.

The machine promises to be of value as an adjunct to other cross infection precautions, especially when certain improvements have been made to the design, such as the addition of shielding to eliminate the danger of explosion if exposed to anaesthetic vapours. It will reduce the bacterial contamination of the air in isolation side-wards, surgical dressing rooms and possibly in such areas as operating theatres and labour wards.

Our thanks are due to the members of the medical and nursing staff of St. Andrew's Hospital whose co-operation made these observations possible, and to the Harris Engineering Company, Ltd. for the loan of a Trion 'Console' electronic air filter.

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A STUDY OF THE EFFECT OF TANNINS ON THE GROWTH OF SULPHATE-REDUCING BACTERIA

By G. H. BOOTH

National Chemical Laboratory, Teddington

SUMMARY: The inhibition by tannins of sulphate-reducing bacteria in pure culture has been shown to be due primarily to the establishment of an unfavourable pH. High concentrations were required to inhibit when the physico-chemical conditions were otherwise favourable.

Following the discovery at York of ancient iron objects in a remarkable state of preservation, Farrer, Biek & Wormwell (1953) suggested that the absence of corrosion could be ascribed to the inhibition of sulphate-reducing bacteria by the presence of tannins in the soil. Laboratory experiments showed that commercially available tannins were inhibitory to the bacteria at concentrations of about 0·01%, but as these experiments were carried out without control of the redox-potential or pH of the medium the effects reported might have been brought about as effectively by, for example, acidifying or aerating the medium and have had no significance with regard to tannins *per se*. A further criticism of the experimental technique employed is that only a short incubation period (5 days) was used. In the examination of soils for the presence of sulphate-reducers it is a common experience for a soil culture to show sudden growth after a very much longer period (20 days or so), depending, for example, on the initial pH and degree of aeration of the sample.

In view of these considerations a further study of the effect of tannins on the growth of pure laboratory cultures of *Desulphovibrio desulphuricans* has been made. Before the systematic investigation reported here, there was evidence (Bufton, unpublished results) that 0·01% of tannic acid was insufficient to prevent the growth of sulphate-reducers under otherwise favourable conditions.

Methods

The culture medium was that of Baars (1934), modified by the addition of yeast extract, 0·1% and $FeSO_4.(NH_4)_2SO_4.6H_2O$, 0·05%. The required amounts of tannins were added to this and it was adjusted in E_h and pH as required. Inoculations were made from a 2 days old culture of *Desulphovibrio desulphuricans* (Hildenborough strain, NCIB 8303), the cultures were incubated at 30° under nitrogen, and examined at intervals for growth. At low concentrations of tannins (below 0·005%) the growth was readily detected by the appearance of pronounced blackening, but at higher concentrations the tubes were so intensely coloured from the outset that this method of judgment was unreliable. In these cases the presence of growth was established by microscopical examination. In all experiments the cultures were set up in test tubes and in duplicate.

The tannins used were: ‘tannic acid’, and myrabolam, mimosa, quebracho and chestnut.

EXPERIMENTS AND RESULTS

Experiments with different concentrations of tannins. Duplicate tubes were set up, containing the tannins at concentrations of 0, 0·0001, 0·0005, 0·001, 0·005, 0·01, 0·05 and 0·1% (w/v) in the culture medium, the pH in each tube being adjusted to 7·2±0·2 before inoculation with 1% (v/v) of the test organism grown in the same medium without tannin.

In all cases, growth had occurred when the cultures were examined after 5 or 6 days.

Experiments at controlled E_h and pH. Tubes identical in every respect with the previous ones except that 0·1% of cysteine hydrochloride was included in the medium to poise the redox-potential (E_h) at about 0·14 V at pH 7, were then set up and inoculated from a culture also containing cysteine. As before, the pH was adjusted to 7·2±0·2 before inoculation. Again, in all cases, growth had occurred when the cultures were examined after 5 or 6 days.

Experiments at varied pH and 0·1% tannin. In all experiments up to this point there had been a common factor in that the pH was adjusted to a value favourable to the growth of the organism. In the next series of experiments the concentration of the tannins was 0·1% and there was no alteration of the pH from the values brought about by the addition of tannin. These, and the pH values with other concentrations, are given in Table 1. Duplicate tubes were again set up, with and without added cysteine, and in addition there were tubes with and without cysteine and without tannin, but with the pH adjusted to 6·6 or to 5·8 by the addition of hydrochloric or phosphoric acids. The tubes were inoculated and examined at intervals during an incubation period of 28 days, and the results are given in Table 2. Growth occurred in 2 days in both controls; but it took 6 or 7 days to appear in tubes

Table 1. *The effect of different concentrations of various tannins on the pH value of a modified Baars' medium**

Concn. of tannin (%)	pH value in medium containing				
	Tannic acid	Myrabolam tannin	Quebracho tannin	Mimosa tannin	Chestnut tannin
0·0001	7·15	7·20	7·30	7·05	7·25
0·0005	7·20	7·20	7·20	7·10	7·30
0·001	7·20	7·15	7·25	7·10	7·35
0·005	7·00	7·02	7·15	7·00	7·28
0·01	6·75	6·78	7·15	6·94	7·20
0·05	6·15	6·15	6·75	6·50	6·35
0·1	5·85	5·85	6·55	6·20	5·68
0·5	5·24	5·24	6·47	6·20	5·20
1·0	4·96	4·90	6·15	6·20	4·91
2·0	4·70	4·53	6·00	6·06	4·72
5·0	4·44	3·98	5·82	6·01	4·39

* Modified by the addition of yeast extract (0·1%) and $FeSO_4.(NH_4)_2SO_4.6H_2O$ (0·05%) but no cysteine.

containing cysteine and where the pH had been lowered by the addition either of tannin or of HCl or H_3PO_4, and did not appear in 28 days in the corresponding tubes without cysteine.

Table 2. *The effect of various additions to a modified Baars' medium on the initiation of growth at* 30° *of* D. desulphuricans

Addition to medium	Time (days) for growth to appear with cysteine		Time (days) for growth after adjustment of pH to 7·2 in tubes negative at 28 days
	Present	Absent	
None	2	2	—
Tannic acid*	6	Not in 28	>25
Myrabolam tannin*	6		>25
Mimosa tannin*	6		6
Quebracho tannin*	6		6
Chestnut tannin*	6		>25
HCl, to pH 6·6	6		6
H_3PO_4, to pH 6·6	6		6
HCl, to pH 5·8	7		>25
H_3PO_4, to pH 5·8	6		>25

* All at 0·1%.

At the end of the 28-day period, to determine whether viable cells were still present in the tubes in which growth had not occurred, i.e. all the tubes without cysteine except the control, these were adjusted to pH 7·2±0·2 and reincubated. The results, which are given in the last column of Table 2, show that viable cells were still present in the tubes where the pH had been around 6·6, but if the pH had been about 5·8 growth did not occur in the 25 day observation period.

Experiments at higher tannin concentrations. The next stage was to examine the effect of E_h and pH adjustment at higher tannin concentrations, but it was considered unnecessary to use conditions in which neither pH nor E_h were favourable. The results with concentrations up to 5% are given in Table 3, sections (a), (b) and (c).

As shown in section (a), in conditions of pH and E_h favourable to the organism all the tannins inhibited growth for at least 28 days only when the concentration was 5%, while at 2% the myrabolam, quebracho and chestnut tannins just allowed growth in that period. At 1% and 0·5% mimosa tannin was the most inhibitory. With favourable pH but unfavourable E_h, as shown in section (b), all the tannins were in general rather more inhibitory, with tannic acid and mimosa tannin the most effective, whilst at favourable E_h but unfavourable pH all the tannins prevented growth for at least 28 days even at 0·5%, as shown in section (c).

To see whether the failure of growth to appear in 28 days in certain tubes of sections (b) and (c) of Table 3 was due to death of the inoculum, those in section (b) were adjusted to E_h 0·14 V and those in section (c) were adjusted to pH 7·2, and reincubated. The adjustment of the section (b) tubes was effected by adding an equal

Table 3. *The effect of adjustment of pH and E_h in a modified Baars' medium on the initiation of growth of* D. desulphuricans *at* 30°

Tannin added	Time (days) for growth with tannin concentrations (%) of						Growth* after adjustment of pH or E_h†, and reinoculation, with tannin concns. (%) of					
	0·0	0·1	0·5	1·0	2·0	5·0	0·25	0·5	1·0	2·0	2·5	5·0
	(a) pH 7·2, E_h 0·14 V.											
Tannic acid	1	8	8	28	>28	>28						
Myrabolam	1	8	14	16	28	>28						
Mimosa	1	8	28	>28	>28	>28						
Quebracho	1	8	8	16	28	>28						
Chestnut	1	8	16	16	28	>28						
	(b) pH 7·2, E_h not adjusted.						(d) pH 7·2, E_h now 0·14 V.					
Tannic acid	1	7	>28	>28	>28	>28	+	+	−		−	
Myrabolam	1	7	7	28	>28	>28			+		−	
Mimosa	1	7	>28	>28	>28	>28	+	+	−		−	
Quebracho	1	7	7	>28	>28	>28		+	−		−	
Chestnut	1	7	7	7	>28	>28			−		−	
	(c) pH not adjusted, E_h 0·14 V.						(e) pH now 7·2, E_h 0·14 V.					
Tannic acid	1	7	>28	>28	>28	>28		+	−	−		−
Myrabolam	1	7	>28	>28	>28	>28		+	+	−		−
Mimosa	1	7	>28	>28	>28	>28		−	−	−		−
Quebracho	1	7	>28	>28	>28	>28		+	−	−		−
Chestnut	1	7	>28	>28	>28	>28		−	−	−		−

* Growth in 14 days: +, present; −, absent. † All tubes of section (b) negative after 28 days were adjusted to E_h 0·14 V, and all tubes of section (c) negative after 28 days were adjusted to pH 7·2.

volume of fresh modified Baars' medium containing 0·2% of cysteine hydrochloride (neutralized), and this caused the tannin concentrations to be reduced by half. However, no growth appeared in any of the tubes after reincubation for 28 days, suggesting that the cells of the original inoculum had died during the first 28 days incubation. The tubes were then reinoculated and incubated for a further 14 days, when the presence or absence of growth was recorded. The results are shown in Table 3, sections (d) and (e), which should be compared with section (a). Section (d) shows that all the tannins were inhibitory at 2·5% and section (d) that they were all inhibitory at 2·0%. All the tannins except myrabolam were inhibitory at 1·0%.

DISCUSSION

From the results presented four general conclusions may be drawn. First, when conditions for growth were otherwise favourable, the presence of tannins had no effect on the growth of *D. desulphuricans* unless the concentration was high (1% in the case of mimosa, 2% with tannic acid and 5% with myrabolam, quebracho and chestnut tannins). Second, in media of favourable E_h but unfavourable pH, inhibition occurred in all cases at 0·5% but not in any case at 0·1% of tannin. Third, in media of unfavourable E_h but at pH 7·2±0·2, inhibition occurred at 0·5% with tannic acid and mimosa, at 1% with quebracho, and at 2% with myrabolam and chestnut tannins. Fourth, no matter whether the E_h was or was not controlled, the effect of tannin addition could be simulated by adding mineral acid to produce pH values equal to those brought about by an inhibitory concentration of tannin. It is thus suggested that the adverse pH brought about by the acidic nature of tannins is the most important factor in the inhibition by these substances of the growth of sulphate-reducers. It is nevertheless apparent that pH is not the only factor, since under favourable conditions for growth the effectiveness of the tannins as inhibitors is not in the order of their acidity. Mimosa tannin, the least acidic of those tested, was apparently the most potent. The range of pH over which growth is restricted is apparently dependent also on whether cysteine has been added to poise the redox-potential of the medium: the presence of cysteine increases the tolerance. Further, there is evidence to indicate that a concentration of tannin sufficient to restrict growth under otherwise unfavourable conditions will prove inadequate if these conditions subsequently became more favourable, even after prolonged exposure of the organism in the unfavourable range.

It appears that even under the most favourable conditions for growth 5% of any of the tannins tested would be effective inhibitors, and such a concentration may be practicable with materials as cheap as tannins; but it is important to realize that in practice this concentration would need to be maintained to ensure continued inhibition. Also, it must be emphasized that the results reported here apply only to pure cultures of *D. desulphuricans* in laboratory liquid media and need not necessarily apply to natural conditions. Protracted field tests on buried metal suitably treated are essential to determine whether or not tannins could be of use in combating underground microbial corrosion.

The author is indebted to Dr. T. White of the Forestal Land, Timber and Railways Co. Ltd. for supplying samples of tannins and to Dr. J. R. Postgate and Mr. K. R. Butlin for advice.

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A MEDIUM FOR THE CULTIVATION OF LACTOBACILLI

By J. C. DE MAN

Nederlands Instituut voor Zuivelonderzoek, Ede, Nederland

M. ROGOSA

National Institute of Dental Research,
National Institutes of Health, Bethesda, Maryland, U.S.A.

and M. ELISABETH SHARPE

National Institute for Research in Dairying,
Shinfield, Reading, England

SUMMARY: An improved growth medium for lactobacilli is described. It supports good growth of lactobacilli generally and also is particularly useful for a number of fastidious strains which grow only poorly in other general media. In addition, tomato juice, a highly variable material, is not required. In a slightly modified form, it can also be used as a basal medium for fermentation tests.

Although several media for lactobacilli have been described, such as the widely used tomato juice medium of Briggs (1953) and Cox & Briggs (1954), it has been found that a number of strains of lactobacilli, belonging to several species, did not grow very well in any of them. Therefore, a need existed for a non-selective medium which would support good growth of lactobacilli in general. Also, it would be desirable to eliminate tomato juice because it is variable and inconvenient to prepare.

Rogosa, Mitchell & Wiseman (1951*a*,*b*) described a selective medium designed especially for the enumeration and isolation of lactobacilli of oral and faecal origin, although it was recognized that it was not satisfactory for certain dairy organisms. Starting from this, it was hoped to develop a new medium in which the lactobacilli of dairy origin would also grow well. A medium of chemically defined composition was not the aim of this study. Rather, it was decided to use commercially obtainable standard ingredients which would be convenient for general use in both routine and research laboratories.

Most of the experiments were performed in two Institutes, the National Institute for Research in Dairying (NIRD) and the Netherlands Institute for Dairy Research (NIZO), using the same strains of lactobacilli and virtually identical methods, and exchanging media components. The results obtained in both Institutes were in agreement. A few of the experiments, by which the composition of the final medium was developed, are reported here.

Materials and Methods

Strains of lactobacilli. The strains used, which are listed in Table 1, were selected as representative strains or because they did not grow satisfactorily in Briggs' medium.

Table 1. *The origin and designation of the* Lactobacillus *species and strains used*

Growth temperature 37°		
Species	Origin*	Designation
L. acidophilus	ATCC	4357
	ATCC	9857
L. bulgaricus	NIZO	3501
L. casei	NIZO	8014
	W	Ku 2
L. fermenti	NIRD	229
	NIRD	F 1
	NIRD	O 10
L. helveticus	NIZO	3524
L. jugurti	NIRD	DR 9
L. lactis	ATCC	8000
	W	Ku 1
L. salivarius	NIH	HO 268
	NIH	HO 66

Growth temperature 30°		
Species	Origin*	Designation
L. bifermentans	NIZO	3903
	NIZO	3905
L. brevis	NIRD	DL 127
	NIRD	V 7
	NIRD	T 1
	NIRD	X 1
L. plantarum	NIZO	3503
Unclassified streptobacteria	NIRD	V 5
	NIRD	A 41

* NIRD, National Institute for Research in Dairying, Shinfield, Great Britain; ATCC, American Type Culture Collection; W, Bakteriologisches Institut der Südd. Versuchs- und Forschungsanstalt für Milchwirtschaft, Freising-Weihenstephan, Germany; NIH, National Institutes of Health, Bethesda, Maryland, U.S.A.; NIZO, Nederlands Instituut voor Zuivelonderzoek, Ede, Holland.

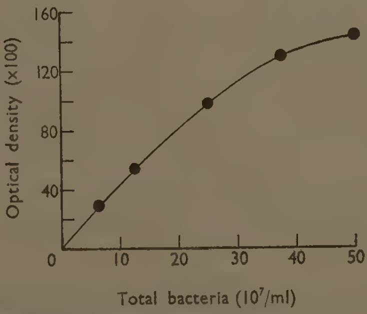

Fig. 1. The relationship between optical density and total (microscopical) bacterial count for lactobacillus strain V 5.

Culturing methods and measurements of growth. In the growth experiments the organisms were cultured in 150×14 mm tubes containing 10 ml of medium. For fermentation tests, tubes with 5 ml of medium were used. Inoculations were always made with 0·1 ml of culture.

For evaluating a growth medium the bacteria were subcultured in it twice, using 48 hr growth periods. Finally, the bacteria were grown for 40 hr in a third subculture, after which growth was measured by determining the optical density of the undiluted culture against a medium blank with an Engel photometer (Kipp, Delft) using filter 66 and a 0·5 cm cell, or with a Hilger Spekker absorptiometer using filter EEL 607. The results are expressed as optical density × 100. Fig. 1 illustrates the relationship between the total number of cells/ml (microscopical counts) and optical density for one of the strains.

As the purpose was the development of a general non-selective growth medium, more optimal growth conditions were achieved at the outset by diminishing the amount of acetate and the use of a higher pH. The final medium was obtained after testing a number of combinations of ingredients and physical conditions, such as pH, different peptones, addition of meat extracts, salts, various carbohydrates, tomato juice and reducing agents. After each experimental change the effect of it on growth was compared with growth on the medium of Briggs (1953), which was used as a standard.

RESULTS AND DISCUSSION

Final growth medium

The medium (referred to below as MRS) as finally composed contained: Oxoid peptone, 10 g; Lab-Lemco (Oxoid), 10 g; Yeast extract (Difco or Oxoid), 5 g; glucose, 20 g; polyoxyethylene sorbitan mono-oleate (Tween 80), 1 ml; K_2HPO_4, 2 g; $CH_3COONa.3H_2O$, 5 g; triammonium citrate, 2 g; $MgSO_4.7H_2O$, 200 mg; $MnSO_4.4H_2O$, 50 mg; distilled water, 1 litre. The pH lies between 6·0 and 6·5 after sterilizing (about 6·2 to 6·6 before). A sterilization time of 15 min at 120° is recommended.

The composition of the new medium is primarily justified by the fact that the selected strains of lactobacilli grew much better in it than in the medium of Briggs (Table 2).

The addition of corn steep liquor did not improve growth. Tomato juice, often used in media for lactobacilli, also was without stimulatory effect, and was therefore considered unnecessary (Table 3). It is an advantage to dispense with tomato juice because it is variable and inconvenient to prepare.

Several kinds of peptone were tried, and of these Oxoid peptone appeared superior to the others. In a final comparison, Difco Tryptone (a casein peptone) gave nearly as good results (Table 4). In further experiments, six different batches of Oxoid peptone were compared: they gave virtually similar growth. When levels of 0·0, 0·2, 0·5, 1·0 and 4% (w/v) were investigated, different strains varied considerably in their growth response. A concentration of 1% (w/v) of Oxoid peptone was chosen, since growth was generally optimal with all the strains investigated. Therefore, 1% (w/v) of peptone was used in all later experiments.

The growth stimulating effect of meat extract in the medium is demonstrated in Table 5.

Rogosa found that with some basal media a mixture of glucose, arabinose and sucrose, instead of only glucose, improved the growth of several fresh isolates of

Table 2. *A comparison of the growth of various lactobacilli in MRS medium with that in Briggs' medium* (1953)

Strain	Growth (OD* × 100)		Strain	Growth (OD* × 100)	
	MRS	Briggs'		MRS	Briggs'
4357	77	26	8000	91	40
3501	75	32	3903	115	4
8014	120	24	DL 127	86	6
229	72	19	V 7	96	8
F 1	110	5	T 1	37	9
O 10	114	27	X 1	107	24
3524	76	46	V 5	132	13
DR 9	68	44			

* OD, optical density.

Table 3. *The effect of tomato juices on the growth of lactobacilli in MRS medium**

Strain	Growth (OD† × 100) in MRS medium with		
	Rijno juice	NIRD juice	No juice
4357	94	105	110
3501	76	66	74
8014	145	140	140
229	24	22	23
3524	80	79	84
DR 9	89	88	88
8000	90	85	92
3903	50	57	95
V 7	112	98	97
V 5	145	140	140

* The media with tomato juice were made with 5% of Rijno tomato juice (a commercial product of Mij De Betuwe, Tiel, Holland), or 10% of tomato juice prepared at NIRD by the method described by Briggs (1953). Correspondingly less water was used in making up the media. † OD, optical density.

heterofermentative organisms (for the resulting medium see Fitzgerald & Jordan, 1953). However, this was not found to be the case with the present medium and the strains studied. Neither was growth better with 4% (w/v) of glucose instead of the 2% (w/v) finally used.

It has been shown that the addition of Tween 80 (Briggs, 1953), citrate (Evans & Niven, 1951) and acetate (Snell, Tatum & Peterson, 1947; Guirard, Snell & Williams, 1946) to media for lactobacilli often results in improved growth. Most of the inorganic ions necessary for growth will be present in sufficient amounts in a complex medium, as used by us. An exception is manganese (Orla-Jensen, 1943; MacLeod & Snell, 1949; Evans & Niven, 1951). Diminishing the amount of Tween 80, or omitting the citrate, acetate or manganese, always resulted in diminished growth for at least several strains. The inclusion of magnesium in the medium is a precautionary measure; it may be unwise to rely on the presence of a sufficient amount of magnesium

Table 4. *A comparison of the growth of various lactobacilli in MRS medium made with Oxoid peptone and Difco Tryptone*

Strain	Growth (OD* × 100) with:		Strain	Growth (OD* × 100) with:	
	Oxoid peptone	Difco Tryptone		Oxoid peptone	Difco Tryptone
4357	92	85	HO 268	111	113
9857	85	97	HO 66	140	140
8014	133	123	3903	120	114
Ku 2	131	125	3905	129	125
229	100	76	V 7	110	98
DR 9	95	83	V 5	140	140
8000	95	94	A 41	145	135
Ku 1	118	110			

* OD, optical density.

Table 5. *The effect of meat extract on the growth of various lactobacilli in MRS medium*

Strain	Growth (OD* × 100) with extract	
	Present	Absent
4357	54	33
3501	65	36
3503	54	38
8014	120	110
F1	99	97
3524	79	77
8000	81	87
3903	107	104
DL 127	136	133
V7	95	92
T 1	71	52
V 5	135	129
A 41	126	121

* OD, optical density.

in the yeast extract or other ingredients, especially as the presence of citrate in the medium increases the amount necessary (MacLeod & Snell, 1947).

No significant differences in growth were encountered within a pH range of 6·1 to 6·6.

Basal medium for fermentation studies

With slight alterations, the MRS medium may also be used for fermentation tests. In this case, the glucose is replaced by 2% (w/v) of the test substrate, the meat extract is omitted since it is known to contain variable amounts of fermentable carbohydrate, and for ease of recognition of the results 0·004% (w/v) of chlorophenol red is added. Nevertheless, in this work not only was the colour noted, but the pH in each tube was also determined.

In an investigation of the influence of initial pH value on the fermentation results, using strains 3531, 3541, DR 9, 229, 3524, F 1, O 10, 3602, V 7, V 5, 3903, DL 127 and T 1 and the substrates arabinose, cellobiose, glucose, lactose, maltose, mannitol, melibiose, sucrose, salicin and trehalose, identical results were obtained with initial pH values of 6·80, 6·20 and 5·75. At lower pH values the results tended to be obtained earlier. For convenience, a pH between 6·2 and 6·5 is recommended. With considerably reduced carbohydrate concentrations (0·2% w/v) a relatively low initial pH can be critical for obtaining consistent fermentation results with some strains, and a pH no higher than 6·5 is suggested.

With the substrates used, no difference was found if the substrates were sterilized together with the basal medium (pH 6·8) or sterilized separately and added later as a 10% (w/v) solution. However, in other media and under different conditions, Rogosa *et al.* (1953) showed that certain substrates, particularly arabinose, xylose, mannose, fructose, galactose and sorbose may require separate sterilization by filtration. Although with the strains tested correct fermentation results were obtained using these heated substrates in the MRS medium, other strains might be encountered giving false positive results from these carbohydrates. The addition of 0·15% of agar to the medium at pH 6·2 did not significantly influence the results.

Positive results could nearly always be recognized after 1 day, and invariably after 4 days. Generally more simple media like those of Orla-Jensen (1919) and Wheater (1955) are also satisfactory for fermentation tests, provided that a high quality peptone is used. The advantage of using the more complex medium is that results are often obtained somewhat sooner and that the number of doubtful reactions is diminished.

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THE ACTION OF NISIN ON SPOILAGE BACTERIA. I. THE EFFECT OF NISIN ON THE HEAT RESISTANCE OF *BACILLUS STEAROTHERMOPHILUS* SPORES

By R. H. THORPE

The Fruit and Vegetable Canning and Quick Freezing Research Association, Chipping Campden

SUMMARY: Spores of two strains of *Bacillus stearothermophilus* were heated for various times both in the absence and presence of nisin and in the latter case both with and without subsequent destruction of the nisin with trypsin. The results obtained on enumeration of the survivors indicated that the reduction of heat resistance which was observed was apparent rather than real and was due to the adsorption of nisin on the spores. When this was removed by means of trypsin the heat resistance was restored almost to normal.

A NEW PRINCIPLE in food preservation—that of the complementary action of the antibiotic subtilin and mild heat—was advanced by Andersen & Michener (1950) and since then there have been numerous papers on the action of antibiotics on bacteria that cause spoilage in canned foods. Considerable attention has also been given to the influence of antibiotics on the heat resistance of bacterial spores and the results, which were often conflicting, have been adequately reviewed by Campbell & O'Brien (1955). O'Brien *et al.* (1956) reported a reduction in the heat resistance of spores of a number of spoilage bacteria when heated in the presence of the antibiotics nisin and subtilin, and Michener (1955), O'Brien & Titus (1955) and O'Brien *et al.* (1956) have put forward evidence that this reduction is probably an apparent one caused by carry-over of the antibiotics into the spore recovery medium.

The successful control of spoilage by *Clostridium thermosaccharolyticum* and *Bacillus stearothermophilus* was reported by Gillespy (1957) in canning trials, using nisin, on beans in tomato sauce and garden peas. The use of nisin to control spoilage bacteria was considered only as an adjunct to the recommended minimum process against *Cl. botulinum.* Michener *et al.* (1959) reported that among the many antibiotics screened for the reduction of heat resistance of the spores of the putrefactive anaerobe *Cl. sporogenes* 3679, nisin and subtilin were the most active. For the purpose of their paper the authors stated that the concept of death or survival was not limited to factors operative solely during the heat treatment.

The present paper, the first paper of a series, reports on the action of nisin on the heat resistance of spores of *B. stearothermophilus.* The aim of the work was to determine, by heating spores in the absence and presence of nisin and subsequently inactivating the nisin with trypsin, whether the reduction of heat resistance reported by previous workers was a true one or only apparent. Evidence is given that the true heat resistance of these spores is not altered by nisin. The work of Campbell & Sniff (1959) was published while this paper was in preparation. They drew similar conclusions.

MATERIALS AND METHODS

Organisms. Two strains of *B. stearothermophilus* (NCA 1518 and NCIB 8224) were used; they were held as stock cultures in this laboratory.

Nisin. Soluble mega unit nisin and standardized nisin were obtained from Aplin & Barrett, Ltd., Yeovil, Somerset. The units are the Reading units (RU) of Mattick & Hirsch (1947) and Berridge (1949), and all the nisin assays were carried out against a standard nisin preparation containing 8,000 RU/g by a modification of the over-night litmus milk assay method of Mattick & Hirsch (1947), using *Streptococcus cremoris* strain 1P/5.

Trypsin. This was Bacto-trypsin (Difco).

Spore recovery medium. This contained: Lab-Lemco, 3 g; yeast extract (Oxoid), 1 g; dextrose, 1 g; peptone (Oxoid), 5 g; tryptone (Oxoid), 5 g; ion agar no. 2 (Oxoid), 8 g; animal charcoal (Oxoid), 8 ml of a 10% suspension; water, 1,000 ml. The pH was adjusted to 6·9–7·0 and the medium sterilized at 15 lb/in^2 for 15 min.

Preparation of stock spore suspension. Plates of carbohydrate-free proteose peptone agar were heavily inoculated from a log phase dextrose-tryptone broth culture, sealed with cellulose self adhesive tape and incubated for 8–10 days at 55°. Sterile water was then poured into the plates and the agar surface lightly rubbed with a sterile glass rod. The resulting suspension was filtered through sterile absorbent cotton and made up to 200–300 ml with sterile water, and this stock suspension was stored at 2°. The spore concentration was estimated by plating decimal dilutions after a heat stimulation (which is necessary to ensure rapid and even germination of *B. stearothermophilus* spores) of 15 min in a steamer. Intermediate stock spore suspensions were then prepared in McIlvaine's buffer solution of pH 6·0.

Final working suspensions. These both contained intermediate stock suspension diluted 1:10 and were made up as follows. Control suspensions (to be heated without nisin) contained 3 ml of well shaken intermediate stock spore suspension and 27 ml of McIlvaine's buffer solution, pH 6·0. Nisin suspensions contained 5 ml of well shaken intermediate stock spore suspension. 5 ml of 100 RU/ml nisin solution and 40 ml of McIlvaine's buffer solution, pH 6·0.

Determination of decimal reduction times. For all heat resistance studies, the spores were heated in 1 ml amounts in sealed ampoules at 115·6° (240°F) in the thermostatically controlled oil bath described by Gillespy (1947). The ampoules, of soft soda glass, were sealed in a bunsen flame after 1 ml of suspension had been introduced from a sterile 10 ml microburette fitted with a hypodermic needle.

Batches of ampoules, held in specially constructed racks, were put into the oil bath at zero time plus 1·75 min, this being the time allowance for heat penetration. The racks of ampoules were lifted out 10 sec before the end of the desired heating periods to allow surplus oil to drain, and were then plunged into a bath of cold paraffin before being thoroughly washed in cold water. To estimate survivors an ampoule was opened and, by applying a low bunsen flame to its base, its contents were expelled into an 8 oz McCartney screw top bottle containing 40 ml of melted yeast extract-dextrose-tryptone (YDT) agar. After mixing by gently rocking the bottle, the medium was distributed between two Petri dishes. These were incubated for 64 hr at 55° (longer incubation did not increase the counts) and the colonies were counted with the aid of a plate microscope, each pair of plates being treated as a single count. Survivor curves were obtained by plotting the logarithms of the mean counts against time and from these the decimal reduction times (D values) were calculated by the method of maximum likelihood.

Inactivation of nisin. The neck of an ampoule containing nisin was broken and 0·5 ml of a 10^{-2} dilution of sterile trypsin introduced from a graduated hypodermic syringe. The ampoule was then resealed, thoroughly shaken and allowed to stand at room temperature (18–20°) for 1 hr before the survivors were estimated.

Determination of the effect of carry-over concentrations of nisin. Ampoules containing 1 ml of control suspension were heated for 5 min at 115·6°. Half the ampoules in each set were discharged into the recovery medium, as described above. The rest were discharged into medium containing nisin, at 0·25 RU/ml for strain NCA 1518 and 0·5 RU/ml for strain NCIB 8224, these being the concentrations which would have been produced as the result of carry-over. The rest of the procedure was as before.

Determination of the effect of adsorbed nisin on heat activated spores. Four 10 ml volumes of well shaken stock spore suspension of NCIB 8224 were centrifuged. To each of two of the sediments, termed A and B, 10 ml of a 10 RU/ml nisin solution were added. The tubes were corked, shaken for 5 min and allowed to stand for a further 85 min at room temperature. To each of the other two sediments, termed C and D, 10 ml of sterile water were added and the tubes shaken and allowed to stand for 85 min. All the tubes were then centrifuged for 15 min at 5,000 rev/min. The spores in tubes A and C were washed with 10 ml of sterile water six times, and those in tubes B and D were first washed three times with 10 ml of a 10^{-2} dilution of sterile trypsin and then three times with 10 ml of sterile water. All four spore sediments were then resuspended, heat stimulated and appropriate dilutions plated in YDT agar for the estimation of survivors.

RESULTS AND DISCUSSION

The effect of nisin on the heat resistance of spores

In these experiments, some of the groups of ampoules which were heated at 115·6° (240°F) for various times contained spore suspension with and without nisin. Those containing nisin were divided into two equal lots after heating and cooling and the nisin in one lot was destroyed with trypsin. Both strains of *B. stearothermophilus* were used, the nisin concentrations being 10 RU/ml with strain NCA 1518 and 10 and 20 RU/ml with strain NCIB 8224. However, nisin inactivation experiments were done only at 20 RU/ml with strain NCIB 8224.

Mean colony counts from heated spore suspensions, together with the variances of the counts, the numbers of replicates, and the variances of the means are given in Tables 1 and 2. The total variance often considerably exceeded the mean and, so far as is known, this accords with general experience for survivor counts. Sufficient data to determine this point is, however, seldom published. The variances of replicate counts of unheated spores (heat stimulated only, Tables 3 and 4) usually did not greatly exceed the means.

Table 1. *The effect of nisin, with and without its subsequent inactivation, on the recovery of spores of* B. stearothermophilus *strain NCA* 1518 *after heating for different times at* 115·6° (240°*F*) *in McIlvaine's buffer solution pH* 6·0

	Statistics of colony counts with 10 RU/ml of nisin:											
	absent, heated for (min)				present, heated for (min)				Present, inactivated, heated for (min)			
	2·5	5·0	7·5	10·0	2·5	5·0	7·5	10·0	2·5	5·0	7·5	10·0
Total colonies	491	306	207	118	101	90	84	65	463	332	221	161
Nos. of plates	10	15	22	28	10	13	19	24	9	14	22	28
Means	49·1	20·4	9·4	4·2	10·1	6·9	4·4	2·7	51·4	23·7	10·0	5·75
Variances	20·32	19·69	27·21	8·54					124·3	28·68	22·81	16·79
Variances of means	2·032	1·313	1·237	0·305					13·81	2·049	1·037	0·600
S.D.s of means	1·426	1·142	1·112	0·552					3·716	1·431	1·018	0·775
Decimal reduction times D (min)	7·03 (S.D. 0·20)								7·56 (S.D. 0·22)			

Table 2. *The effect of nisin, with and without its subsequent inactivation, on the recovery of spores of* B. stearothermophilus *strain NCIB* 8224 *after heating for different times at* 115·6° (240°*F*) *in McIlvaine's buffer solution pH* 6·0

	Statistics of colony counts with nisin:							
	absent, spores heated for (min)				present (20 RU/ml), inactivated, spores heated for (min)			
	2·5	5·0	7·5	10·0	2·5	5·0	7·5	10·00
Total colonies	1847	844	479	304	1814	928	523	312
Nos. of plates	14	26	40	57	15	26	38	51
Means	131·93	32·46	11·98	5·33	120·93	35·69	13·76	6·12
Variances	564·2	38·26	14·13	5·37	198·21	65·02	17·32	6·75
Variances of means	40·3	1·47	0·353	0·0942	13·21	2·42	0·456	0·132
S.D.s of means	6·348	1·212	0·594	0·097	3·635	1·556	0·675	0·363
Decimal reduction times, D (min)	5·24 (S.D. 0·07)				5·37 (S.D. 0·07)			
	present (10 RU/ml), spores heated for (min)				present (20 RU/ml), spores heated for (min)			
	2·5	5·0	7·5	10·0	2·5	5·0	7·5	10·0
Total colonies	844	502	289	192	109	47	64	32
Nos. of plates	12	21	27	48	12	12	34	25
Means	70·33	23·90	10·70	4·00	9·08	3·925	1·88	1·28

The relationships between the logarithms of the mean values of survivors and time are shown in Figs. 1 and 2. The two strains behaved similarly except that strain NCIB 8224 was less sensitive to nisin than strain NCA 1518. The presence during heating of 10 RU/ml nisin in the suspending medium greatly reduced the recovery counts of strain NCA 1518 compared with the controls. This concentration of nisin had little effect on strain NCIB 8224, but with 20 RU/ml even greater reductions were observed. However, when the spores were treated with trypsin after having been heated in the presence of nisin, the recovery counts were similar to those from controls heated in the absence of nisin. Indeed, statistically these two sets of recovery counts belonged to the same population.

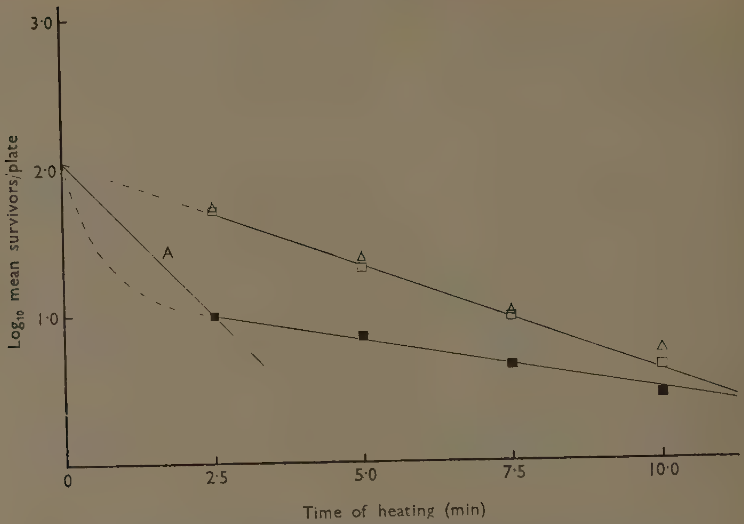

Fig. 1. Survival curves for *B. stearothermophilus* strain NCA 1518 heated at 115·6° (240°F) in McIlvaine's buffer solution at pH 6·0.
Open squares, no nisin present; full squares, 10·0 RU/ml present; open triangles, 10·0 RU/ml nisin present, subsequently inactivated with trypsin.

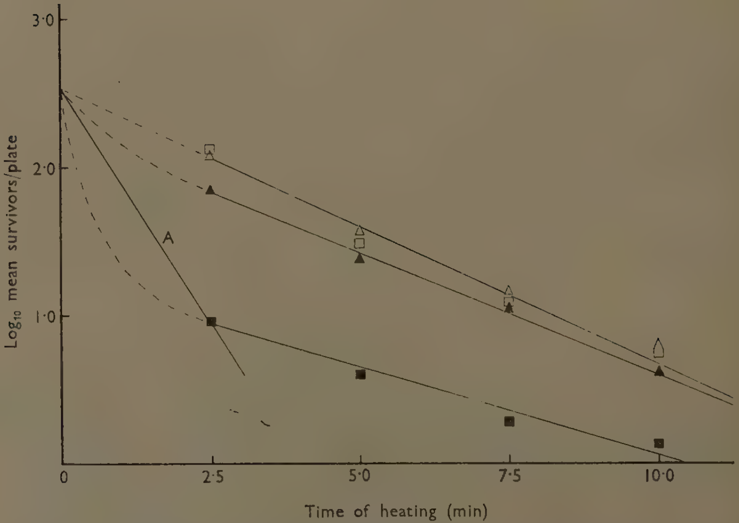

Fig. 2. Survival curves for *B. stearothermophilus* strain NCIB 8224 heated at 115·6° (240°F) in McIlvaine's buffer solution at pH 6·0.
Open squares, no nisin present; full triangles, 10·0 RU/ml nisin present; full squares, 20·0 RU/ml nisin present; open triangles, 20·0 RU/ml nisin present, subsequently inactivated with trypsin.

The weight to be attached to a point on a survivor curve is proportional to the total number of survivors counted for that point, and this should be borne in mind when estimating by eye the slope of a line passing through the points. Although in the present work the total survival counts for each time of heating (Tables 1 and 2) were not even approximately equal, an attempt had been made to make them more nearly so by increasing the number of replicate plates according to the time of heating, so far as was practicable. From most published data it is not possible to estimate the weights of points on survivor curves. As Figs. 1 and 2 show, there was a linear relationship between the logarithm of the mean colony count/plate and time over the range studied. Best fitting straight lines and decimal reduction times (D values) have been calculated, by the method of maximum likelihood, from the results for spores heated in the absence of nisin and in the presence of nisin with subsequent destruction. These D values are very similar. If the nisin was not destroyed after heating, the heat sensitivity curve was markedly altered. Although linear over the time range studied, there was evidently a greatly reduced survival especially in the earlier stages. If the initial population values indicated by the lines for the spores heated without nisin are used in conjunction with the results for only one heating time, e.g. the 2·5 min values (lines A in Figs. 1 and 2), the D values for spores heated with nisin are much less than those for spores heated without nisin. This type of result could be interpreted to give some of the findings of Michener *et al.* (1959). A possible explanation of the shape of the graph obtained when spores were heated with nisin would be that less nisin was available to act on the spores after the longer heating times.

The fact that, when the nisin was inactivated with trypsin after the spores had been heated, the survival curve was the same as for spores heated without nisin indicates that the nisin treatment had not affected the heat resistance of the spores themselves. Accordingly the greater apparent heat sensitivity of spores heated with nisin was probably due to nisin either carried over into the recovery medium with the inoculum or adsorbed on the spores, or both, and it is further indicated that this nisin acted on the germinating spore, or during its outgrowth. Tests to establish the roles of carried-over and adsorbed nisin were then made.

The effect of carry-over concentrations of nisin

If the carry-over of free nisin was alone the cause of the lowering of the survival counts, then concentrations of 0·25 and 0·5 RU/ml in the recovery medium ought, respectively, to inhibit or greatly reduce the numbers of colonies of strains NCA 1518 and NCIB 8224 developing from spores heated in the absence of nisin. However, no significant reductions were obtained when these concentrations of nisin were used (Table 3) and it was concluded that the carry-over of nisin was not important.

The effect of adsorbed nisin on heat stimulated spores

When spores of strain NCIB 8224 were treated with nisin and washed with water before heat stimulation, their ability to yield colonies was greatly impaired (treatments A and C, Table 4). This indicates that nisin was adsorbed by these spores.

Table 3. *The effect of 'carry over' concentrations of nisin on colony formation by heat stimulated spores of* B. stearothermophilus

	Statistics of colony counts of strain			
	NCA 1518		NCIB 8224	
	No nisin	0·25 RU/ml	No nisin	0·5 RU/ml
Total colonies	287	277	377	385
Nos. of plates	12	12	12	12
Means	23·93	23·08	31·42	32·08
Variances	24·81	30·08	58·81	49·72
Variance of means	2·07	2·51	4·90	4·14
S.D.s of means	1·42	1·58	2·21	2·03

Table 4. *The effect of exposure to nisin on the ability of spores of* B. stearothermophilus *NCIB* 8224 *to form colonies after different subsequent treatments*

	Statistics of colony count, adsorbed nisin			
	Present, spores washed in		Absent, spores washed in	
	water (A)	trypsin and water (B)	water (C)	trypsin and water (D)
Total colonies	99	387	389	405
Nos. of plates	10	10	10	10
Means	9·9	38·7	38·9	40·5
Variances	10·1	46·90	39·43	42·30
Variances of means	1·01	4·69	3·94	4·23
S.D.s of means	1·00	2·17	1·98	2·06

However, if the spores were washed with trypsin after exposure to nisin, the counts were of the same order as those of the controls (treatments B, C and D, Table 4). These results support the suggestion that nisin is not sporicidal for this strain of *B. stearothermophilus* and that the lowering of the recovery counts occasioned by nisin treatment was due to inhibition either of spore germination or of outgrowth by adsorbed antibiotic.

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THE EFFECT OF CHLORTETRACYCLINE ON EVISCERATED CHILLED CHICKENS COMMERCIALLY PROCESSED IN GREAT BRITAIN

By ELIZABETH A. WALKER and J. H. TAYLOR
Cyanamid of Great Britain Ltd., London

AND

J. G. DAVIS
Dr. J. G. Davis and Partners, 9, Gerrard Street, London, W.1

SUMMARY: Thirty broiler chickens of known weight and history were processed simultaneously under normal conditions in each of three poultry packing stations, visually assessed to be operating at 'ideal', 'good', and 'poor' levels of hygiene. The eviscerated carcases were bisected, cooled to 3° in chilled air, and distributed between three treatment groups which were dipped for 30 min in chilled water containing 0, 10 and 20 p/m of chlortetracycline respectively. During storage at 3° for 3 weeks, microbiological and sensory examinations were undertaken. At all levels of hygiene, the application of the antibiotic significantly delayed the onset of spoilage; the differences between the effects of 10 and 20 p/m were small and generally insignificant. Bacterial counts of 10^6/cm^2 of skin were obtained from the controls on the 7th day of storage whereas similar counts on the treated groups were not reached until the 14th day. 'Off' odours developed in the control groups between the 5th and 7th days and in the treated groups between the 10th and 14th days. The nature of the spoilage flora acquired during processing appeared to be associated with the duration of storage.

The normal spoilage of eviscerated poultry takes place on the skin and on the exposed surfaces of the visceral cavity. It is mainly due to the proliferation of a wide variety of micro-organisms, particularly bacteria, and is characterized by an offensive odour and visible 'bacterial slime' when the microbial counts reach 10^7–10^8/cm^2 of skin (Walker & Ayres, 1956). The rate of spoilage can be partially controlled by adequate hygiene, which thereby reduces the microbial load acquired during processing, and also by storing the processed carcases at temperatures which delay the rate of microbial proliferation.

Kohler, Miller & Broquist (1955) first reported that chlortetracycline effectively inhibited the growth of poultry spoilage organisms *in vitro* and that eviscerated carcases dipped in a solution containing 10 p/m of chlortetracycline remained fresh during storage at 3° for 21 days. Further work has confirmed and extended these observations (Ziegler & Stadelman, 1955; Ayres *et al.* 1956; McVicker *et al.* 1958; Barnes & Shrimpton, 1958). Shannon & Stadelman (1957) and Shrimpton (1957) demonstrated that chlortetracycline was ineffective at unrefrigerated temperatures, while Ayres *et al.* (1956) reported that a notable change in the nature of the spoilage flora resulted when the normal level of chlortetracycline (10 p/m) was used. Representatives of *Pseudomonas* and *Achromobacter* constituted almost the entire flora within 2 days of the commencement of storage at 4·4°, and during storage the proportion of yeasts increased. In 1955, the commercial use of chlortetracycline was permitted in the U.S.A., tolerance limits for permissible residues being defined (Regulation, 1955).

Optimum benefit from the treatment requires a satisfactory standard of hygiene. As wide variations exist in the methods and conditions of commercial poultry processing, some of which are unsuited to the use of an antibiotic, a detailed survey of 20% of the registered poultry processing plants of Great Britain was carried out to form a basis for the present investigation. The results indicated that a significant proportion of the processing was being done under conditions which would permit the use of chlortetracycline to extend the subsequent storage life (Taylor & Walker, 1959). The purpose of the work reported below was to determine the effects of chlortetracycline on the storage life of chilled chickens processed under varying conditions of hygiene related to normal practice in processing plants in Great Britain in 1958.

Materials and Methods

Processing and treatment of birds

Selection of processing stations. Three stations were selected (by visual inspection) according to a visual assessment of the standard of hygiene adopted. In all stations the floors were concrete, the walls were painted, the drainage and lighting were adequate and the poultry, after slaughter and plucking, were cooled with mechanically circulated air at temperatures between −1° and +4°, in insulated chambers. In station 1, adequate space was available for the quantity of poultry handled, and the killing and plucking sections were partitioned from the evisceration section. Mechanically operated plucking units, overhead conveyor equipment, an evisceration trough and appliances for rinsing the eviscerated carcases were installed. The floors and all equipment were washed thoroughly at the conclusion of each day's operation. These conditions were considered to facilitate processing at a high standard of hygiene. This station (the National Institute of Poultry Husbandry) functioned for demonstration and teaching purposes and processed relatively few birds; the degree of bacterial contamination was therefore likely to be comparatively low. On this basis it was designated an 'ideal' station. In station 2 the conditions and methods of processing and the degree and frequency of cleaning, and consequently the standard of hygiene, were similar to those in the 'ideal' station; but as it functioned commercially and processed 2,000 birds/day the degree of contamination was likely to be greater and it was designated a 'good' commercial station. In station 3 the space available for processing was inadequate. A thermostatically controlled scald tank and a mechanical plucking unit were installed, but wooden tables were used for evisceration and for stacking the carcases between the various operations. There was excessive handling of the carcases which, after evisceration, were wiped with an unrinsed cloth. Thorough cleaning of the plant was undertaken only at weekly intervals. Approximately 500 birds were processed daily. The standard of hygiene was thus considered to be comparatively low and to be representative of a 'poor' commercial operation.

Birds. On each of two occasions 15 broiler chickens which had been reared on deep litter and weighed $3\frac{1}{2}$–$3\frac{3}{4}$ lb were distributed simultaneously to each of the three stations. Those sent to the 'ideal' station were obtained from an adjoining experimental farm and had had a broiler ration containing 7 g of penicillin/ton. Those sent to the 'good' and 'poor' commercial stations had been reared together and had been fed on a commercial broiler ration containing 9 g of chlortetracycline/ton.

Processing and grouping of experimental birds. Before the experiment was started, up to 200 birds were processed in both the 'good' and 'poor' commercial stations. The experimental birds were stunned, bled via the jugular vein for 1 min, scalded at 53·3–54·4° for 30 sec, mechanically plucked, hand stubbed and then eviscerated. The left and right sides were labelled and the carcases bisected. The half carcases were either rinsed in water or wiped with a cloth according to normal practice, then suspended on a metal stand and cooled by air to an internal body temperature of 4·4°. The giblets were not included. In order to compensate for differences in the microbial count which might exist between birds, the half carcases were distributed according to a randomized incomplete block design between three groups; there were thus 10 unmatched left and right halves in each group at each station.

Application of chlortetracycline and storage. On each occasion at each station sufficient water at 4·4° was put into each of three 20 gal galvanized iron containers to give a ratio by weight of carcases to water of 1:2. To two of these were added freshly prepared solutions of chlortetracycline at levels which provided, respectively, 10 and 20 p/m in the water and carcases, and to the third an equal volume of water was added. The 10 half carcases of a group were placed in the containers and submerged for 30 min, then momentarily drained, enclosed separately in polythene bags, packed with a small quantity of ice in an insulated container and transported to the laboratory.

On arrival at the laboratory each half carcase was transferred to a sterilized cellophane box so that the peritoneal surface was uppermost, and then stored at 3±1°.

Microbiological examination

The workers who did the examinations were not aware of the kind of treatment which had been applied. On the skin over the breast of each half carcase seven areas of 1 in^2 were delineated and each was swabbed on one occasion of examination according to a randomized sequence. A moistened sterile gauze swab was rubbed for 5 sec over the prescribed area and returned to 20 ml of quarter-strength Ringer's solution, which was shaken. The procedure was repeated on each half carcase. After thorough shaking decimal dilutions were prepared and variable but known volumes, according to the colonies expected, were delivered from sterilized dropping pipettes as described by Davis & Bell (1959) on to the appropriate agar medium and spread evenly. Preliminary experiments were done to determine the degree of dilution, volume of inoculum, type of medium and time of incubation required for a satisfactory experimental technique.

Colony counts at 18°. These were done on Yeastrel-milk agar (Wilson *et al.* 1935) incubated for 4 days, the carcases being examined on 1, 3, 7, 10, 14, 17 and 20 days storage. In a preliminary trial it was found that the addition of minced chicken to this medium had no effect on the counts obtained.

Colony counts at 5°. These were done on Yeastrel-milk agar incubated for 14 days. The carcases were examined on days 1, 7, 14, and 20.

Yeasts and moulds. These were counted on glucose-yeast agar adjusted to pH 3·5 with citric acid (Davis, 1931, 1958), after incubation at 18° for 6 days, the carcases being examined on 1, 7, 14, and 20 days storage. This medium gave counts similar to those obtained on beer wort agar.

Sensory examination

Individual observations were made on each half carcase on 3, 6, 8, 10, 13, 15, 17, and 20 days storage. On each occasion, each of four members of a trained panel gave an assessment of the nature and extent of the microbial growth on each half carcase and of the odour. A fresh chicken was provided to serve as a standard of freshness and the odours were classified as shown in Table 1.

Table 1. *The classification of the odours of chicken carcases during storage at* 3°

	Score	Description of odour		Score	Description of odour
'Flesh' odours	1*	'Sweet' odour characteristic of freshly killed chicken	'Off' odours	4	Distinctive initial faint odour on discrete areas
	2*	Characteristic odour of flesh		5	Distinctive faint odour over entire carcase
	3*	'Flesh' odour well developed		6	Offensive odour
				7†	Very offensive odour

* Carcases marketable as the fresh commodity. † When carcases reached this score they were examined microbiologically and then discarded.

RESULTS

Statistical analysis showed that comparisons of the colony counts for the same bird were no more accurate than comparisons between different birds. No increase in accuracy was gained therefore as a result of the balance with regard to half carcases. The two replicates were shown to be of comparable accuracy and are reported together. The statistical analyses were carried out by using the logarithms of the mean counts (subsequently reconverted) in order to reduce the effects of extreme observations, which were however infrequent, and to provide homogeneity of variance.

Microbiological examination

Comparisons between the control and treated groups were not possible after the fourteenth day of storage, as most of the control carcases had by then been discarded because of their offensive condition.

Colony counts at 18°. Table 2 shows the relationship between the level of hygiene during processing and the increase in the bacteria during storage. On the first and third days highly significant differences ($P < 0.001$) reflecting the levels of hygiene existed between the mean counts of the control carcases; but after 14 days these differences were no longer apparent. Table 2 also shows that, independent of the level of hygiene, bacterial growth was delayed significantly ($P < 0{\cdot}001$) by 10 and 20 p/m of chlortetracycline. There was little difference between the effects of these concentrations in the early part of the storage period, but a significant difference ($P < 0{\cdot}001$) became apparent on the 14th and 20th days when the 10 p/m group had double the count of the 20 p/m group. However, this difference was largely due to the response at the 'poor' level of hygiene.

Table 2. *The effect of chlortetracycline (CTC) treatment on the colony count on Yeastrel-milk agar at* 18° *of chicken carcases stored at* 3°

Period of storage at 3° (days)	CTC concn. (p/m)	Colony count*/cm² of skin at hygiene levels rated			S.R.†	Mean colony count*/cm² of skin	S.R.†
		'Ideal'	'Good'	'Poor'			
1	0	$5{\cdot}0\times10^{2}$	$2{\cdot}5\times10^{3}$	$1{\cdot}3\times10^{4}$	2·4	$2{\cdot}5\times10^{3}$	1·7
	10	$4{\cdot}0\times10^{2}$	$1{\cdot}9\times10^{3}$	$7{\cdot}9\times10^{3}$		$1{\cdot}8\times10^{3}$	
	20	$3{\cdot}0\times10^{2}$	$1{\cdot}8\times10^{3}$	$8{\cdot}0\times10^{3}$		$1{\cdot}6\times10^{3}$	
3	0	$1{\cdot}0\times10^{4}$	$4{\cdot}6\times10^{4}$	$1{\cdot}7\times10^{5}$	2·7	$4{\cdot}3\times10^{4}$	1·8
	10	$2{\cdot}3\times10^{3}$	$1{\cdot}5\times10^{4}$	$2{\cdot}6\times10^{4}$		$9{\cdot}7\times10^{3}$	
	20	$2{\cdot}0\times10^{3}$	$1{\cdot}6\times10^{4}$	$2{\cdot}9\times10^{4}$		$9{\cdot}8\times10^{3}$	
7	0	$4{\cdot}7\times10^{6}$	$6{\cdot}0\times10^{6}$	$1{\cdot}2\times10^{7}$	2·8	$6{\cdot}9\times10^{6}$	1·9
	10	$1{\cdot}0\times10^{4}$	$4{\cdot}1\times10^{5}$	$4{\cdot}1\times10^{4}$		$5{\cdot}5\times10^{4}$	
	20	$1{\cdot}0\times10^{4}$	$2{\cdot}3\times10^{5}$	$4{\cdot}1\times10^{4}$		$4{\cdot}6\times10^{4}$	
10	0	$1{\cdot}1\times10^{8}$	$2{\cdot}4\times10^{8}$	$1{\cdot}7\times10^{8}$	3·0	$1{\cdot}6\times10^{8}$	1·9
	10	$5{\cdot}0\times10^{4}$	$8{\cdot}5\times10^{5}$	$5{\cdot}0\times10^{4}$		$1{\cdot}3\times10^{5}$	
	20	$7{\cdot}0\times10^{4}$	$4{\cdot}5\times10^{5}$	$4{\cdot}0\times10^{4}$		$1{\cdot}0\times10^{5}$	
14	0	$4{\cdot}9\times10^{8}$	$4{\cdot}7\times10^{8}$	$5{\cdot}0\times10^{8}$	3·9	$4{\cdot}9\times10^{8}$	2·2
	10	$1{\cdot}4\times10^{6}$	$6{\cdot}5\times10^{6}$	$3{\cdot}4\times10^{6}$		$3{\cdot}2\times10^{6}$	
	20	$9{\cdot}9\times10^{5}$	$4{\cdot}6\times10^{6}$	$1{\cdot}2\times10^{6}$		$1{\cdot}8\times10^{6}$	
17	0	‡	‡	‡		‡	
	10	$5{\cdot}7\times10^{6}$	$6{\cdot}3\times10^{6}$	$1{\cdot}8\times10^{6}$	3·9	$4{\cdot}0\times10^{6}$	2·2
	20	$3{\cdot}4\times10^{6}$	$7{\cdot}1\times10^{6}$	$1{\cdot}5\times10^{6}$		$3{\cdot}3\times10^{6}$	
20	0	‡	‡	‡		‡	
	10	$1{\cdot}1\times10^{7}$	$2{\cdot}2\times10^{7}$	$2{\cdot}0\times10^{7}$	3·8	$1{\cdot}7\times10^{7}$	2·2
	20	$4{\cdot}7\times10^{6}$	$2{\cdot}3\times10^{7}$	$5{\cdot}0\times10^{6}$		$8{\cdot}4\times10^{6}$	

* Geometric mean of 2 replicate experiments each with 10 half carcases at each level of treatment. † S.R., significance ratio. When the ratio of the larger number to the smaller exceeds the significance ratio, the difference between the two values is significant ($P < 0{\cdot}001$). ‡ Carcases discarded because of their offensive condition.

Colony counts at 5°. Table 3 shows that on the control carcases the growth of bacteria at 5° bore the same general relationship to the level of hygiene in processing as did growth at 18°. The former were significantly inhibited by the chlortetracycline treatment, but a significant difference ($P < 0{\cdot}05$) between the two concentrations was apparent only on the 14th day. This was again mainly due to the difference at the 'poor' level of hygiene.

Yeasts. The rate of proliferation of the yeasts is shown in Table 4. The incidence of yeast contamination was higher in the 'good' commercial station than in the other stations. During storage yeasts developed on all the birds, although marked increases on the treated carcases occurred only after the 7th day.

Table 3. *The effect of chlortetracycline (CTC) treatment on the colony count on Yeastrel-milk agar at 5° of chicken carcases stored at 3°*

Period of storage at 3° (days)	CTC concn. (p/m)	Colony count*/cm² of skin at hygiene levels rated			S.R.†	Mean colony count*/cm² of skin	S.R.†
		'Ideal'	'Good'	'Poor'			
1	0	$3\cdot 0 \times 10^{3}$	$4\cdot 4 \times 10^{3}$	$1\cdot 3 \times 10^{4}$	3·9	$5\cdot 5 \times 10^{3}$	2·2
	10	$1\cdot 9 \times 10^{3}$	$1\cdot 7 \times 10^{3}$	$8\cdot 1 \times 10^{3}$		$3\cdot 0 \times 10^{3}$	
	20	$1\cdot 6 \times 10^{3}$	$3\cdot 1 \times 10^{3}$	$7\cdot 0 \times 10^{3}$		$3\cdot 3 \times 10^{3}$	
7	0	$6\cdot 8 \times 10^{6}$	$1\cdot 1 \times 10^{7}$	$1\cdot 4 \times 10^{7}$	3·9	$1\cdot 0 \times 10^{7}$	2·2
	10	$7\cdot 0 \times 10^{3}$	$1\cdot 0 \times 10^{5}$	$6\cdot 0 \times 10^{3}$		$1\cdot 6 \times 10^{4}$	
	20	$6\cdot 0 \times 10^{3}$	$7\cdot 7 \times 10^{4}$	$4\cdot 0 \times 10^{3}$		$1\cdot 2 \times 10^{4}$	
14	0	$3\cdot 6 \times 10^{8}$	$4\cdot 4 \times 10^{8}$	$2\cdot 7 \times 10^{8}$	4·2	$3\cdot 5 \times 10^{8}$	2·4
	10	$9\cdot 2 \times 10^{5}$	$2\cdot 1 \times 10^{6}$	$1\cdot 7 \times 10^{6}$		$1\cdot 5 \times 10^{6}$	
	20	$7\cdot 5 \times 10^{5}$	$1\cdot 4 \times 10^{6}$	$4\cdot 4 \times 10^{5}$		$7\cdot 7 \times 10^{5}$	
20	0	‡	‡	‡		‡	
	10	$9\cdot 7 \times 10^{6}$	$2\cdot 3 \times 10^{7}$	$1\cdot 4 \times 10^{7}$	5·0	$1\cdot 4 \times 10^{7}$	2·5
	20	$5\cdot 2 \times 10^{6}$	$1\cdot 8 \times 10^{7}$	$3\cdot 9 \times 10^{6}$		$7\cdot 0 \times 10^{6}$	

* Geometric mean of 2 replicate experiments each with 10 half carcases at each level of treatment. † S.R., significance ratio. When the ratio of the larger number to the smaller exceeds the significance ratio, the difference between the two values is significant ($P < 0\cdot 001$). ‡ Carcases discarded because of their offensive condition.

Table 4. *The effect of chlortetracycline (CTC) treatment on the yeast count on glucose-yeast agar at 18° of chicken carcases stored at 3°*

Period of storage at 3° (days)	CTC concn. (p/m)	Colony count*/cm² of skin at hygiene levels rated			S.R.†	Mean colony count*/cm² of skin	S.R.†
		'Ideal'	'Good'	'Poor'			
1	0	$<3\cdot 0 \times 10^{2}$	$<5\cdot 0 \times 10^{3}$	$<7\cdot 0 \times 10^{2}$		Not calculated	
	10	$<3\cdot 0 \times 10^{2}$	$<5\cdot 0 \times 10^{3}$	$<7\cdot 0 \times 10^{2}$			
	20	$<3\cdot 0 \times 10^{2}$	$<5\cdot 0 \times 10^{3}$	$<7\cdot 0 \times 10^{2}$			
7	0	$1\cdot 0 \times 10^{4}$	$2\cdot 1 \times 10^{5}$	$1\cdot 5 \times 10^{4}$	3·3	$3\cdot 2 \times 10^{4}$	2·0
	10	$7\cdot 0 \times 10^{3}$	$2\cdot 9 \times 10^{5}$	$1\cdot 0 \times 10^{4}$		$2\cdot 7 \times 10^{4}$	
	20	$1\cdot 0 \times 10^{4}$	$1\cdot 8 \times 10^{5}$	$1\cdot 3 \times 10^{4}$		$2\cdot 8 \times 10^{4}$	
14	0	$4\cdot 0 \times 10^{4}$	$3\cdot 7 \times 10^{5}$	$1\cdot 7 \times 10^{5}$	6·8	$1\cdot 3 \times 10^{5}$	3·1
	10	$7\cdot 6 \times 10^{5}$	$4\cdot 4 \times 10^{6}$	$1\cdot 2 \times 10^{6}$		$1\cdot 6 \times 10^{6}$	
	20	$5\cdot 5 \times 10^{5}$	$3\cdot 2 \times 10^{6}$	$9\cdot 0 \times 10^{5}$		$1\cdot 2 \times 10^{6}$	
20	0	‡	‡	‡		‡	
	10	$1\cdot 3 \times 10^{6}$	$1\cdot 0 \times 10^{7}$	$4\cdot 4 \times 10^{6}$	2·7	$3\cdot 9 \times 10^{6}$	1·8
	20	$1\cdot 4 \times 10^{6}$	$1\cdot 1 \times 10^{7}$	$2\cdot 9 \times 10^{6}$		$3\cdot 6 \times 10^{6}$	

* Geometric mean of 2 replicate experiments each with 10 half carcases at each level of treatment. † S.R., significance ratio. When the ratio of the larger number to the smaller exceeds the significance ratio, the difference between the two values is significant ($P < 0\cdot 001$). ‡ Carcases discarded because of their offensive condition.

Moulds. Notable changes in the mould counts were not apparent until the 14th day, and the increases at this time were greatest on the control carcases, most of which had counts in excess of 10^{4}–10^{5}/cm^{2}. The percentage of treated birds with counts of this order was much smaller and the two concentrations of antibiotic had similar effects.

Sensory examination

Appearance. The mean times at which microbial growth was first visible on the half carcases are shown in Table 5. Bacterial and yeast colonies, which could not be differentiated, were first apparent on the pleural and peritoneal membranes of the control halves. Within 2–4 days they extended over a large portion of the carcase and subsequently coalesced to produce a typical 'bacterial slime'. This was so with 65% of the control group by the 10th day. Treatment with chlortetracycline significantly delayed the appearance of colonies but there were no apparent differences between the concentrations. Moulds became the dominant visible flora on the treated carcases between the 14th and 20th days.

Table 5. *The effect of chlortetracycline on the time of appearance of macroscopic microbial growth on chicken carcases stored at 3°*

Concn. of chlor-tetra-cycline (p/m)	Mean* and S.D.† of storage time (days) till colonies seen at hygiene levels rated			Grand mean duration of storage, with S.D.† (days)
	'Ideal'	'Good'	'Poor'	
0	7·1 ± 1·4	6·4 ± 0·8	6·6 ± 0·9	6·7 ± 1·0
10	14·8 ± 2·6	9·8 ± 2·5	14·4 ± 2·7	13·0 ± 2·6
20	14·7 ± 2·7	10·3 ± 2·7	15·4 ± 1·4	13·5 ± 2·2

* Each treatment group at each level of hygiene contained 10 half carcases, and there were 2 replicate experiments. † Standard deviation.

Table 6. *The effect of chlortetracycline (CTC) on the odour assessments of chicken carcases stored at 3°*

Storage time (days)	CTC concn. (p/m)	Mean* assessments† at hygiene levels rated			Grand means for groups
		'Ideal'	'Good'	'Poor'	
3	0	1·2	1·2	1·2	1·2
	10	1·1	1·0	1·0	1·1
	20	1·1	1·1	1·2	1·1
7	0	2·6	4·4	3·3	3·4
	10	1·9	2·0	1·3	1·7
	20	1·2	2·0	1·6	1·6
10	0	4·8	6·4	5·6	5·6
	10	2·3	2·5	2·3	2·4
	20	2·2	2·8	2·4	2·5
14	0	5·9	7·0	6·6	6·5
	10	3·2	4·5	3·2	3·6
	20	3·5	4·4	3·0	3·6
17	0	6·8	‡	‡	‡
	10	4·3	5·9	4·2	4·8
	20	4·3	5·6	4·0	4·6
20	0	‡	‡	‡	‡
	10	5·0	6·6	5·0	5·5
	20	5·0	6·6	4·8	5·5

* Each treatment group at each level of hygiene contained 10 half carcases, and there were 2 replicate experiments. † Odour assessment: 1·0–3·0, fresh; 3·0–5·0, faint but distinctive 'off' odours; > 5·0, strong and offensive 'off' odours. ‡ Carcases discarded because of their offensive condition.

Odour. The mean odour scores of all the groups are shown in Table 6 and the mean storage times before a distinct 'off' odour (score 5) could be detected are given in Table 7. The storage life of the control and treated carcases varied with, but was not relative to, the level of hygiene. Chlortetracycline at both levels used delayed the onset of 'off' odours and thereby appreciably extended storage life. The differences between the control and treated groups were highly significant ($P < 0{\cdot}001$). In one replicate, the left halves remained fresh (score 3 and under) for 0·6 days longer than the right halves, this difference being significant ($P < 0{\cdot}05$).

Table 7. *The effect of chlortetracycline (CTC) on the storage life, assessed by odour, of chicken carcases stored at 3°*

Carcase condition and odour score*	Hygiene level	Mean† days of storage to reach condition given at CTC levels (p/m) of			S.E.‡	Extensions in mean storage life (days) due to CTC treatment at levels (p/m) of	
		0	10	20		10	20
Fresh, score <3	'Ideal'	7·8	13·8	13·6	±0·5	6·0	5·8
	'Good'	5·6	11·3	10·9		5·7	5·3
	'Poor'	6·7	13·7	14·1		7·1	7·4
	Means	6·7	12·9	12·9	±0·3	6·2	6·2
Faint 'off' odour, score 4	'Ideal'	9·1	16·1	16·5	±0·6	7·0	7·4
	'Good'	6·7	13·2	13·4		6·5	6·7
	'Poor'	8·0	17·0	17·4		9·0	9·4
	Means	7·9	15·4	15·8	±0·3	7·5	7·8

* Description given in Table 1. † Each treatment group at each level of hygiene contained 10 half carcases, and there were 2 replicate experiments. ‡ S.E., standard error of group of 9 mean storage times or of 3 mean values at the separate treatment levels.

DISCUSSION

The swabbing technique used has been shown by Ayres *et al.* (1950) to produce results comparable to those obtained by the excision and homogenization of a standard area of skin and underlying tissue. It has certain advantages over the 'rinse' technique described by Kohler *et al.* (1956) in that fewer carcases are required and that several measurements can be derived from the same carcases during storage. When this experiment was done the results of Barnes & Shrimpton (1958) were not available.

The results obtained agree with those reported by other workers in that the application of either 10 or 20 p/m of chlortetracycline significantly delayed the proliferation of spoilage bacteria, thereby approximately doubling the storage life of the treated carcases. Although the two concentrations tested caused no significant difference in effect, it is unlikely that a level much below 10 p/m would produce optimum results (Kohler *et al.* 1955). Ayres *et al.* (1956) and Wells & Stadelman (1958) reported that, because bacteria were suppressed by 10 p/m of chlortetracycline applied under laboratory conditions, yeasts were encouraged. Simpson *et al.* (1959) demonstrated that under commercial conditions of processing the numbers of yeasts on carcases treated with chlortetracycline did not approach those of bacteria and that spoilage was primarily due to the bacterial flora. The results obtained in the present experiment indicate that the degree of contamination by yeasts may vary markedly between stations and that the application of chlortetracycline may permit the growth of yeasts during the middle rather than the early part of the storage period.

The nature of the spoilage flora appears to be an important factor in the potential storage life of carcases, as evidenced by the relationship between the onset of spoilage and the size of the bacterial and yeast populations acquired during processing at each station. The subjective methods used for selecting the three stations proved to be reliable only in that the size of the initial contamination on the control carcases was related to the respective levels of hygiene as assessed visually, but not with regard to the nature of the flora. The differences in the floras could not be wholly attributed to the conditions of rearing, nor to the nature of the feed, as the birds used in the 'good' and 'poor' commercial stations were reared simultaneously and given identical rations which contained small quantities of chlortetracycline. Irrespective of these wide variations in commercial processing and their consequent effect upon the storage potential of the carcase, the treatment with chlortetracycline was highly effective.

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THE EFFECT OF CHLORTETRACYCLINE ON EVISCERATED FROZEN CHICKENS COMMERCIALLY PROCESSED IN GREAT BRITAIN

BY ELIZABETH A. WALKER AND J. H. TAYLOR
Cyanamid of Great Britain Ltd., London

SUMMARY: Twenty broiler chickens of known history were processed, bisected, randomly distributed between two groups and then cooled for 2 hr in water and ice. One group was treated with 10 p/m of chlortetracycline. After wrapping, the carcases were blast frozen and stored, first at − 15° for 5 months, and then at 3°. During the latter period sensory examinations were done. Treatment with chlortetracycline delayed the appearance of visible microbial growth from a mean of 6·2 days in the controls to 7·8 days, and the onset of 'off' odours was also delayed, from a mean of 6·1 days in the controls to 8·5 days. It appeared that poultry meat which had been frozen and thawed was more highly perishable than fresh chilled poultry meat.

AN EXTENSION in the storage life of chilled eviscerated chickens due to the application of chlortetracycline has been clearly established and recently confirmed experimentally under commercial conditions of poultry processing in Great Britain (Walker, Taylor & Davis, 1960). In this country, however, a large proportion of eviscerated chickens are deep frozen for periods of up to 6 months. Windlan *et al.* (1956) demonstrated with turkeys that the effects of applying 10 p/m of chlortetracycline were evident during the chilled storage period following frozen storage for 30 days; an extension of storage at 1·5° of 7–10 days was obtained.

The purpose of the present experiment was to evaluate the application of chlortetracycline to eviscerated chickens which, after treatment, were deep frozen for 24 weeks, thawed and held in chilled storage. The investigation was carried out at a commercial station where the methods of processing and the standard of hygiene were those of good commercial practice in Great Britain.

MATERIALS AND METHODS

Preparation of half carcases. Twenty broiler chickens of 3½–3¾ lb weight were used. They had been reared on deep litter and had received a commercial broiler ration containing 10 g of penicillin/ton until the 6th week and thereafter chlortetracycline and oxytetracycline, each at 10 g/ton. The birds were all sent together to a packing station of the 'good commercial' type described by Walker *et al.* (1960) on a day when 1,000 chickens had already been processed. They were stunned, bled, scalded at 53·3° for 2 min, mechanically plucked, eviscerated, labelled, bisected and rinsed in cold water. The 40 half carcases were distributed randomly between

2 groups so that each contained an equal number of unmatched left and right halves. The giblets were excluded. Four additional half carcases were similarly treated and used for temperature measurements.

Treatment with chlortetracycline, freezing and storage. Two 20 gal galvanized iron containers each received specified quantities of water and ice, and into one of these a solution of chlortetracycline was mixed to give a final concentration of 10 p/m of carcase, water and ice, these being present in equal proportions by weight. The 20 half carcases of each group were submerged in a container for 2 hr, then removed, momentarily drained, enclosed individually in polythene bags, blast frozen at –20° for 5 hr, placed in containers and stored under identical conditions at approximately –15° for 24 weeks. Before freezing, a meat thermometer was inserted vertically to a depth of 4 in. through the cut surface into the breast tissue of the 4 additional half carcases. All the carcases were then transported in insulated containers to the laboratory, where they were stored at 3±1°. They thawed, and the internal body temperature reached 1° within 24–30 hr. This was designated day 0 of chilled storage. The half carcases were then removed from their wrappers and transferred to separate cellophane boxes; storage at 3±1° was continued.

The experimental treatment which had been applied to each half carcase was unknown to the operators involved in the subsequent procedures.

Sensory examination. The carcases, which were examined by a trained panel comprising the same six members throughout, were assessed in a manner similar to that described by Walker *et al.* (1960).

RESULTS

Visible microbial growth

The visual observations on the nature of the microbial growth were confirmed by cultural tests on nutrient agar, MacConkey's agar and wort agar. Bacterial and yeast colonies were first apparent on the pleural and peritoneal membranes of the control halves at 6·2±0·6 days and within 2 more days they had spread over the entire surface of the visceral cavity. As they coalesced typical 'bacterial slime' began to form. Colonies first appeared on the carcases treated with chlortetracycline at 7·8±0·6 days, but their subsequent spread was slower and 'bacterial slime' did not occur until the 13th day. However, faint mould growth was observed between 10 and 13 days.

Table 1. *The effect of chlortetracycline on the odour assessment of chicken carcases during storage at 3° following deep frozen storage*

Chlortetracycline (p/m)	Mean* odour assessment† during storage at 3° on days						
	1	3	6	8	10	13	15
0	1·2	1·5	2·9	5·3	6·6	7·0	‡
10	1·2	1·4	2·2	2·8	4·2	6·0	7·0

* Each level of treatment was used on 20 half carcases. † Assessment: 1·0–3·0, fresh; 3·0–5·0, faint but distinctive 'off' odours; >5·0, strong and offensive 'off' odours (Walker *et al.* 1960). ‡ Carcases discarded due to their offensive condition.

Odour development

As shown in Table 1, the rate of development of odours was similar in both groups. Chlortetracycline treatment extended the mean storage life from 6·1 to 8·5 days, the difference being highly significant ($P < 0{\cdot}001$).

DISCUSSION

Although the increase in storage life due to the treatment with 10 p/m of chlortetracycline prior to deep frozen storage was highly significant, it was appreciably less than that obtained when carcases were stored at 3° without previous freezing (Walker *et al.* 1960). It is possible that the initial condition of the half carcases was different in the two experiments, but against this is the fact that in both investigations the first signs of spoilage were observed about the 6th day. It was notable that in the present experiment the control carcases deteriorated more rapidly than did the fresh chilled carcases in the previous experiment, and that advanced spoilage, evidenced by 'bacterial slime' and an offensive odour, was apparent by the 8th day. A similar degree of deterioration was not observed in the 'chilled' chickens until the 14th day. It thus appears that poultry meat which has been held in frozen storage and thawed is more perishable than the fresh, chilled commodity.

In view of the fact that Windlan *et al.* (1956) found a significant difference between the keeping qualities of turkeys dipped for 2 hr and 22 hr in 10 p/m of chlortetracycline, it may be that dipping for more than 2 hr would lead to a greater extension of the storage time.

The authors wish to express their thanks to the Directors and Staff of the packing station which co-operated in the experiment, to the Staff of the Meat Section of the National College of Food Technology for participating in the Sensory Assessment Panel, to Mr. C. W. K. Simmons for undertaking the microbiological examinations and to Mr. M. H. Quenouille for the statistical analysis of the results.

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MICROBIAL SULPHIDE PRODUCTION FROM SULPHATE-ENRICHED SEWAGE SLUDGE

By K. R. BUTLIN, SYLVIA C. SELWYN
and D. S. WAKERLEY

National Chemical Laboratory, Department of Scientific and Industrial Research, Teddington, Middlesex

SUMMARY: A laboratory process for the microbial production of sulphide from sewage sludge enriched with sulphate has been further developed. Semicontinuous fermentations yielded 0·9–1·0% S^{2-} using a 10-day replacement time (RT) and 0·8% S^{2-} with a 5-day RT. Yields at 37° were slightly higher than at 30°; at 43° and above yields were much lower. A mixture of 70% N_2 and 30% CO_2 was successfully used as sweeping gas to remove H_2S; other proportions of these gases were less efficient. Stripping H_2S from the sludge in an external column was as efficient as passing the stripping gas through the bulk of the fermenting sludge.

A semicontinuous laboratory process for the anaerobic microbial production of sulphide from raw sewage sludge enriched with calcium sulphate has already been described (Butlin, Selwyn & Wakerley, 1956). Pilot plant trials at the Northern Outfall Works, Beckton (Report, 1958) confirmed the laboratory results and stimulated work not only on improving the process but on problems which would arise in larger scale operations. This paper describes the effect on sulphide yields of using shorter replacement times (RTs) and of varying the fermentation temperature over the range 30–55°; the use of mixtures of N_2 and CO_2 instead of CH_4 and CO_2 for sweeping out H_2S, and the effect of gaseous contaminants likely to be present in recirculated sweeping gas, were also examined. A method for stripping H_2S from the fermenting sludge in an external column was developed.

Materials and Methods

Percentages are w/v except where otherwise stated. Yields of sulphide are given as percentages (wt S^{2-} produced as H_2S/vol. of raw sludge used in sulphide fermentations).

Sludges. Raw sewage sludges from the West Middlesex Main Drainage, Isleworth, were used in all experiments. Fresh supplies were obtained at 3–4 week intervals, because the sludge deteriorated slowly even when stored at 4°. Wherever possible the same stock was used throughout an experiment in order to prevent variation of results through variation in the nature of the sludge. In long series of experiments it was necessary to use sludges of varying quality, and controls using these same sludges were essential. All sludges, including those enriched with precipitated calcium sulphate ($CaSO_4.H_2O$, approx.), were homogenized in a Waring blender before use.

Mixtures of N_2 *and* CO_2. Carbon dioxide was introduced into a cylinder containing N_2 at atmospheric pressure to give a pressure which was in the same proportion to the final pressure (1500 lb/in^2) as the percentage of CO_2 desired. Thus, for a 70% N_2 plus 30% CO_2 mixture, CO_2 was added to give a pressure of about 450 lb/in^2. After the cylinder had stood overnight to allow the temperature to equilibrate, the pressure was readjusted to 450 lb/in^2 by expelling or adding CO_2. Oxygen-free N_2 was then introduced to give a pressure of about 1500 lb/in^2. Finally, the gas mixture was analyzed for CO_2 and any necessary adjustment made.

Gas analyses. The H_2S in the final effluent gas was estimated by passing measured volumes through a 1% solution of cadmium acetate; precipitated CdS was determined by adding excess 0·5 N I_2, then conc. HCl, and titrating excess I_2 against 0·1 N $Na_2S_2O_3$, with starch as indicator. Estimation of CO_2 was by absorption in 30% KOH solution in an Orsat apparatus, and CH_4 or N_2 were estimated by difference.

Estimation of sulphide in sludge. For soluble sulphide, 50 ml quantities of sludge were refluxed for 30 min in a stream of 'oxygen-free' nitrogen (<10 p/m O_2), the H_2S in the gas stream being absorbed in 1% cadmium acetate and estimated iodometrically. For total sulphide, the samples were acidified with an equal volume of conc. HCl before refluxing. Insoluble sulphide was estimated by difference.

Settlement tests. Samples of sludge (100 ml) were allowed to settle in graduated cylinders for 24 hr at room temperature (*c.* 20°). The volume of supernatant liquid was quoted as a percentage of the total volume.

pH. Routine pH determinations were carried out colorimetrically; they were checked periodically by potentiometric determinations.

Effect of shorter replacement time on sulphide yields

Our previous paper described semicontinuous sulphide fermentations operated (with good yields) with 20-day and 40-day RTs, i.e. each day 1/20th or 1/40th of the fermenting sludge was withdrawn and replaced with an equal volume of raw sludge. Industrially it might be preferable to use a shorter RT even at the cost of smaller sulphide yields. Experiments were therefore carried out to determine the optimal yields of H_2S obtainable with 10-day and 5-day RTs. Previous work with a 20-day RT (Butlin, Selwyn & Wakerley, 1956) showed that the yield of H_2S depended on the rate of sweeping and that the highest yields were obtained when the rate was such that the gas after sweeping contained 5–6% H_2S. Thus for comparison it was necessary to observe this condition in the experiments using 10-day and 5-day RTs.

Apparatus and procedure. Sulphide fermentations (1 l. capacity) were set up and operated as previously described (Butlin, Selwyn & Wakerley, 1956). The larger quantities of sweeping gas used were provided from either one or two 10 l. methane fermentations fed into a 15 l. gas reservoir and metered through needle valves to the sulphide fermentations. Rotameters (Rotameter Manufacturing Co., Croydon) were used to measure the gas flow rates. A diagrammatic representation of the gas collection system is shown in Fig. 1. The effluent gases from the sulphide fermentations were collected over acidified saturated brine in a graduated 20 l. aspirator (A).

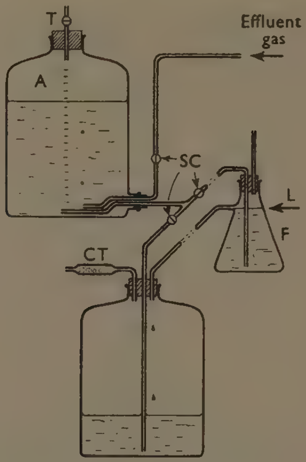

Fig. 1. Diagrammatic representation of the gas collection system.

The pressure of gas in the effluent gas line and in the aspirator is controlled by the level (L) of acidified brine in a 2 l. Buchner flask (F). The gas in the aspirator is measured at atmospheric pressure by clipping off the effluent gas line and raising the flask (F). Gas samples are obtained by raising the flask (F) still further to bring the gas pressure above atmospheric and expelling gas through the tap (T). Gas is removed from the aspirator (A) by connecting the tap (T) to a water vacuum pump, reversing the positions of the 3 screw clips or artery forceps (SC), and opening the tap. Traces of H_2S which escape from solution into the air space of the lower vessel are removed in a carbon trap (CT). The experiments were carried out in a room maintained at 30±2°.

In this series of experiments a fermentation with a 20-day RT, which had previously given optimal yields with a given rate of sweeping (Butlin, Selwyn & Wakerley, 1956) was changed over to a 10-day RT. The rate of sweeping was then increased by trial and error until the effluent gas contained 5–6% H_2S. The change from a 10-day to a 5-day RT was made with a 7-day RT as an intermediate stage. The rate of sweeping was again increased until the effluent gas contained 5–6% H_2S. All experiments were continued for at least one complete replacement period after the fermentation had become steady, i.e. at least 20 days for a 20-day RT.

Effect of fermentation temperature on sulphide production

Apparatus and procedure. The apparatus and techniques for conducting the semicontinuous sulphide fermentations were the same as described in the previous section. Yields were determined over a temperature range of 30–55°; semicontinuous fermentations (all with 20-day RTs) were used for temperatures of 30–50° but a batch fermentation was employed at 55°. Fermentations at 30° were housed in a room maintained at 30±2°, and those at higher temperatures in an incubator maintained within ±1° of the required temperature. In the batch fermentation at 55° (see also Butlin, Selwyn & Wakerley, 1956), nitrogen was blown through a mixture of 50 ml of raw sludge, 8 g of $CaSO_4.H_2O$ and 80 ml of 'active' sulphide sludge from a 50° semicontinuous fermentation, inoculated with a culture of *Clostridium nigrificans* and material from a 'hot' compost heap. The H_2S produced was trapped in 1% cadmium acetate and estimated iodometrically. A control batch fermentation was set up at 30°.

Acclimatization. The general scheme adopted was to start with a fermentation at 30° and gradually raise the temperature by steps of 1–2°; yields were determined at certain chosen temperatures when the production of H_2S had become steady. Because it was impossible to use the same stock of raw sewage sludge throughout the long series of experiments, controls at 30° were set up for each temperature investigated. At temperatures up to 40°, no difficulty was experienced in obtaining steady production of H_2S within 1–2 days. Above 40°, however, longer periods were required before steady conditions were reached and it was found necessary to raise the temperature very slowly. To initiate a fermentation at 43°, sludge fermenting at 40° was incubated first at 41·5° for 3 days and then at 43° for a week before the daily sulphide yield became steady. For the 50° fermentation, fermenting sludge at 40° was incubated at progressively higher temperatures over a period of 10 days; it took a further 24 days for H_2S production to become steady. All experiments were continued for at least 20 days after stable conditions had been reached.

Use of mixtures of N_2 and CO_2 as sweeping gas

Hitherto, both in laboratory semicontinuous experiments and pilot plant trials, H_2S had been swept from the fermenting sludge by 'sludge gas' (70% CH_4 plus 30% CO_2) produced during anaerobic digestion of raw sludge containing no added sulphate. This gas is inflammable and, although suitable for small scale operations, might well be dangerous on a large scale. A suitable non-inflammable sweeping gas, if cheap enough, would be preferred.

A process for the recovery of elemental sulphur from mixtures of H_2S, N_2 and CO_2 (Catchpole & Cuddington, 1954 *a,b,c*, 1955) gives rise to flue gases consisting mainly of N_2 and CO_2 which would be available for continuous use in sweeping out H_2S from the fermenting sludge by recirculation in a closed system. As previously described (Butlin, Selwyn & Wakerley, 1956) N_2 alone was successfully used for stripping H_2S from batch fermentations, but its efficiency compared with a mixture of 70% CH_4 and 30% CO_2 in semicontinuous fermentations was unknown. Experiments were therefore carried out to compare N_2 or N_2 and CO_2 mixtures with a mixture of 70% CH_4 and 30% CO_2. Experiments with CO_2 alone were also included, since it was soon found that the presence of CO_2 considerably affected the yields.

Apparatus and procedure. The apparatus (1 l. fermenting capacity) and techniques for carrying out the semicontinuous fermentations were as previously described, except that N_2 and CO_2 mixtures in various proportions were employed for sweeping H_2S from the fermenting sulphide sludges. The main experiments were carried out in a series starting with N_2, and continuing with gases containing increasing quantities of CO_2. A separate sulphide fermentation swept with 70% CH_4 and 30% CO_2 was operated with the same RT as a control. To guard against variation in quality of the sludge the control was fed from the same (but varying) samples of sludge as used in the main experiments. Throughout the series 10-day RTs were used, except for the N_2 and CO_2 experiments (20-day RTs).

Sulphide yields and the total residual sulphide in the fermenting sludges were determined daily for all fermentations including controls. pH values of the fermenting sludges were also determined. All experiments were carried on for at least 20 days after stable conditions had been reached.

Effect of possible contaminating gases in mixtures of N_2 and CO_2 used as sweeping gas

There was a possibility that, in certain working conditions, air, sulphur dioxide and carbonyl sulphide might be introduced (probably intermittently rather than steadily) into the flue gases mentioned in the previous section. Since it was intended to recycle these gases continuously through the fermenting sludge the effect of such contaminants on the sulphide yield was investigated.

Apparatus and procedure. Two sulphide fermentations, one as control, were operated with a 10-day RT at 30°, and swept with 12 l./day of a mixture of 70% N_2 and 30% CO_2. Gaseous SO_2, contained in a separating funnel, was introduced into the sweeping gas of one fermenter by displacement with mercury. Additions were made for 3 hr each day to give 4% SO_2 (v/v) in the sweeping gas on the first day, 8% on the second, 16% on the third and 32% on the 7 succeeding days. The fermentation was then swept with uncontaminated 70% N_2 and 30% CO_2 for several days to remove all traces of SO_2, after which air was added to the sweeping gas for 3 hr daily in the same manner as for SO_2, except that, as 32% air partially inhibited the fermentation, concentrations above 16% were not used. After a further period (2–3 days) of sweeping with uncontaminated gas, carbonyl sulphide was introduced. The calculated quantity of a saturated solution of KCNS was added to a cooled mixture of 3 vol. conc. H_2SO_4 and 4 vol. of H_2O in a 250 ml flask through which the sweeping gas passed. The aim was to introduce about 2, 4 and 8% into the sweeping gas over daily periods of 6 hr on successive days, but the amounts actually added only approximated to these. Yields of H_2S were determined daily.

External stripping of H_2S from fermenting sludge

Hitherto, both in laboratory and pilot plant experiments, H_2S had been recovered by sweeping gas continuously through the sludge in the fermenter. Experience with the pilot plant indicated that it might be difficult to distribute the sweeping gas efficiently throughout the sludge in a large fermenter containing thousands of tons of sludge. An alternative method was suggested by the common practice of circulating methane-producing sludges from the bottom to the top of the fermenter through an outside pipe: with sulphide sludges it might be possible to strip H_2S from the sludge while it was passing through this exterior column. Moreover, this exterior column could be kept at a higher temperature than that of the fermenter, which might assist the release of H_2S. It might also be more efficient to sweep the stripping gas against the flow of sludge rather than with it. These problems were investigated as described below. The work was not completed owing to events beyond our control, but sufficient was accomplished to show the efficacy of the method.

Apparatus and procedure. The apparatus is illustrated in Plate 1. Sludge, magnetically stirred, was continuously circulated from the fermenter (2 l. capacity) through the external stripping column ($24 \times \frac{1}{2}$ in.) and back to the fermenter by a Sigmamotor peristaltic pump (V. A. Howe & Co., London, W.11) in which 'Tygon' vinyl tubing (grade R-3603, $\frac{3}{8}$ in. bore; V. A. Howe & Co., London, W.11) was used. Elsewhere rubber tubing with glass connections was used ($\frac{1}{2}$ in. between fermenter and column, and $\frac{3}{4}$ in. for the return). The stripping column was jacketted and maintained at the required temperature by water circulated by a 'Circotherm' (Shandon Scientific Co. Ltd., London, S.W.7). Sweeping gas (70% N_2 and 30% CO_2 from a cylinder) was blown into the sludge at the base of the column through a non-return valve made by cutting a slit in a rubber teat. The rate of sweeping throughout the experiments was 16 l./day. The metering of the sweeping gas (see right of Plate 1) and collection of the effluent gas (Fig. 1 and Plate 1, bottom left) were as previously described. A wide tube ($1\frac{1}{2}$ in.) at the top of the stripping column allowed any froth to break, and a 2 l. bottle connected to the tube provided an overflow in case of blockage of the return tubes. The rate of circulation of the sludge was maintained at 4–8 circulations/day, and was estimated by observing the time taken for sludge to fill the graduated glass tube (Plate 1, top left of column) when the flow was interrupted by a screw clamp fitted below the graduated tube. A pressure control system (Fig. 2), based on a device described by Hall (1955), was introduced below the stripping column to compensate for any temporary blockages in the column. The operation of this system is as follows: sweeping gas under a pressure determined by the depth of liquid (45% $Ca(NO_3)_2$, S.G. 1.42) in the vent tube (V) and by the setting of the needle valve (N) is blown through a cut rubber teat (T) into the stripping column (SC). The rate of gas flow is measured by a Rotameter (R). Temporary blockages in the stripping column cause fluctuations in the pressure required to force gas into the column. Any rise or fall in the back pressure causes the depth of liquid in the vent tube (V) to increase or decrease so that the pressure drop across the needle valve (N), and hence the gas flow, remains constant. A cut-out device for the circulating pump in case of complete blockage was placed in the sludge circuit on the output side of the pump (Fig. 3). This operates as follows: any pressure increase due to blockage causes the 'Symel' silicone elastomer sleeving (S) of 12 mm bore and 1 mm wall (H. D. Symonds & Co. Ltd., Kingston Hill, Surrey) to bulge and push against the spring-loaded brass plate (P) fitted over a window ($\frac{3}{8} \times \frac{3}{8}$ in.) cut in the brass sheathing tube (B). The brass plate (P) in turn presses on the arm (A) of a microswitch (Type CR/RL, Burgess Products Co. Ltd., Gateshead) which actuates a press-button operated direct starter (Allen West & Co. Ltd., Brighton) and switches off the pump.

During initial experiments the apparatus was housed in the open laboratory. The fermenting sludge was maintained at 30° by a heating mat (240Ω) operated at a reduced voltage (80 V) and controlled by a type T.S.1 thermostat (Sunvic Controls Ltd., Harlow, Essex) clamped vertically to the outside of the fermenter and insulated from external temperatures by a foam rubber sheath. Later, because it was suspected that sulphide yields were reduced by cooling of the sludge during circulation through other parts of the apparatus, the whole apparatus was transferred to a room maintained at 30±2°. In these conditions, cooling of the fermenter was found to be necessary when the stripping column was kept at 40 or 45°, and a thermostatically controlled water cooling system was devised. Coils of rubber tubing were wrapped round the fermenter and the attached thermostat. A 250 W car engine radiator water heater (G. Bray & Co. Ltd., Leeds 2) was placed in the input side of the water circuit and connected through a relay with the thermostat. Water was passed slowly through the heater and cells and its temperature, and that of the fermenter, were controlled by the action of the thermostat on the heater.

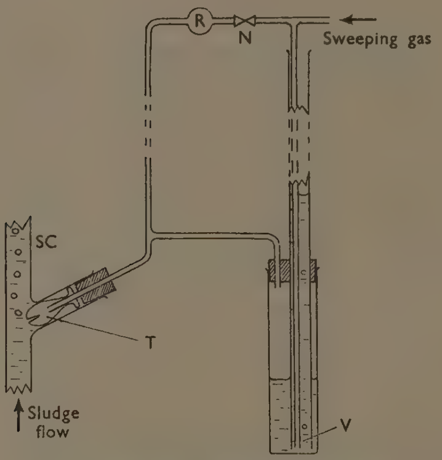

Fig. 2. Diagrammatic representation of the pressure control system.

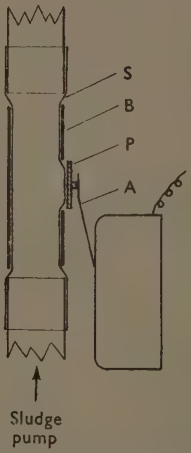

Fig. 3. Diagrammatic representation of the circulating pump cut-out device.

Each day 200 ml were removed from the circulating sludge and replaced by 200 ml of raw sludge containing 8% $CaSO_4.H_2O$ added through a glass funnel. The sludge was removed by means of a wide 200 ml tube attached to the return tube and normally kept upright as shown in Plate 1; sludge flowed into the tube when it was lowered.

For experiments using countercurrent flows of sludge and sweeping gas, the stripping column was placed below the fermenter. Sludge flowed through a glass side entry and down the column, while gas passed up. The gas separated from the sludge at the side entry and a splash head prevented entry of froth into the gas line. The sludge was pumped from the base of the stripping column back to the fermenter.

Controls were run throughout the series of experiments and consisted of fermentations (10-day RTs) in which H_2S was removed by sweeping gas continuously through the whole mass of fermenting sludge at 30° in the fermenter (Butlin, Selwyn & Wakerley, 1956). The controls were fed from the same stocks of sludge as those used in the main experiments and sweeping gas was supplied from the same cylinder. Sulphide yields were determined daily in both sets of fermentations, and all experiments were carried on for at least 20 days.

RESULTS AND DISCUSSION

Effect of shorter replacement time on sulphide yields

Table 1 gives the yields of sulphide obtained with RTs of 20, 10 and 5 days and different rates of sweeping which, except in one instance, gave 5–6% H_2S in the effluent gas mixture. The results show clearly that as the RT was shortened (i.e. the quantity of sludge used/day increased) the quantity of sulphide produced/day increased considerably though not in proportion, and the percentage yield decreased.

Table 1. *Influence of replacement time on sulphide yields at* 30±2° *and pH* 7·1±0·1

Replacement time (days)	Rate of sweeping (l./day)	H_2S concn.*† in final gas (% w/v)	Yield* of S^{2-} as	
			g/day	% w/v of raw sludge
20	8·0	6·0	0·65	1·30
10	8·0	7·5	0·82	0·82
10	12·0	6·0	0·97	0·97
10	12·0	5·5	0·90	0·90‡
5	22·0	5·5	1·66	0·83‡

* Average results over the period of steady conditions for each replacement time.

† H_2S removed from 1 l. sludge + 8% $CaSO_4.H_2O$ by sweeping with a mixture of 70% CH_4 and 30% CO_2.

‡ The same stock of sludge was used in these experiments.

Economically, the increased daily yield, together with the more rapid treatment of the sludge, might be preferred to a greater percentage yield from the sludge. The 5-day RT fermentation had one serious disadvantage in that settlement tests on the final sludge showed that the amount of removable water was only 25% compared with 50% for the final sludge from a 10-day RT fermentation, probably because digestion was incomplete. This poor settlement would increase the cost of disposal of the final sludge and for this reason alone the 10-day RT is to be preferred.

Effect of fermentation temperature on sulphide production

Yields of sulphide were approximately the same throughout the temperature range 30–41° (Table 2) but above 41° there was a marked drop to 0·1% at 43°; repeated attempts failed to increase this value. At 50° the yield was 0·35% compared with 1% in the 30° control. Batch fermentations at 55°, after repeated subcultures into fresh raw sludge, produced only 0·7% (w/v) of S^{2-} in 23 days compared with 1% in 7 days from the control batch fermentations at 30°.

It was concluded that there was no advantage in carrying out the sulphide fermentation above 30°.

Use of mixtures of N_2 and CO_2 as sweeping gas

The yield (97%) from the fermentation swept with 73% N_2 and 27% CO_2 was almost the same as that obtained when a mixture of 70% CH_4 and 30% CO_2 was

Table 2. *Influence of temperature on sulphide yields*

Temperature (°C ± 1)	Rate of sweeping (l./day)	Yield* of S^{2-}†‡ (% w/v of raw sludge)
30	8	1·3
37	8	1·1 (0·95)
37	8	1·5 (1·45)
40	8	1·1 (1·15)
41	8	1·15 (1·20)
43	8	0·1 (1·20)
50	5	0·35 (1·00)

* Average of 20-day period.

† S^{2-} removed as H_2S from 1 l. of sewage sludge + 8% $CaSO_4.H_2O$ by sweeping with a mixture of 70% CH_4 and 30% CO_2; replacement time 20 days; pH 7·1 ± 0·1.

‡ Numbers in brackets are the values for control fermentations at 30° swept with 8 l. of gas/day.

used; other mixtures and N_2 alone gave significantly lower yields, while CO_2 alone gave less than half (Table 3). On the other hand, the figures for residual sulphide in the fermenting sludge show that, as would be expected, the efficiency of removal of H_2S depends on the concentration of CO_2 in the sweeping gas. Thus, although the residual values for 6, 27 and 71% CO_2 are not strictly comparable (since the same stock of sludge was not used throughout) the trend was obvious. Despite this increase in efficiency of removal of H_2S with increase in CO_2 concentration, the yields of H_2S swept from the sludge, in comparison with those obtained with 70% CH_4 and 30% CO_2, decreased with CO_2 concentrations of 46% and over. This means that at these concentrations the use of CO_2 partially inhibited the production of sulphide from sulphate to an extent not explicable by the slight decrease in pH.

Table 3. *Comparison of the efficiencies of various N_2 and CO_2 mixtures with a mixture of 70% CH_4 and 30% CO_2 as sweeping gas for the removal of H_2S from sulphide fermentations at 30 ± 2°*

Composition* of sweeping† gas	Replacement time	pH‡	Yield§ of S^{2-} (% w/v of raw sludge)	Residual§ S^{2-} in sludge (mg/100 ml)
N_2	20	7·2	0·73	0·67
Control	20	7·0	0·81	0·44
94% N_2 + 6% CO_2	10	7·2	0·76	0·87
Control	10	7·0	1·02	0·77
84% N_2 + 16% CO_2	10	7·1	0·90	0·78
Control	10	7·0	0·98	0·76
73% N_2 + 27% CO_2	10	7·0	0·61	0·44
Control	10	7·0	0·63	0·45
54% N_2 + 46% CO_2	10	6·9	0·64	0·45
Control	10	7·0	0·78	0·63
29% N_2 + 71% CO_2	10	6·9	0·65	0·51
Control	10	7·0	0·92	0·73
CO_2	20	6·8	0·60	0·30
Control	20	7·0	1·25	0·49

* Controls composed of 70% CH_4 and 30% CO_2.

† Rate of sweeping 8 l./day. ‡ ±0·1.

§ Average results obtained over a 20-day period from 1 l. sewage sludge + 8% $CaSO_4.H_2O$.

As a result of these experiments it was decided to recommend the use of 70% N_2 and 30% CO_2 as sweeping gas in any large scale process.

Effect of possible contaminating gases in mixtures of N_2 and CO_2 used as sweeping gas

Sulphide yields were unaffected by sweeping the fermenting sludges with a mixture of N_2 and CO_2 containing up to 32% SO_2 for 3 hr daily, or up to 8% carbonyl sulphide for 6 hr daily, over periods of 10 days. Yields were normal with sweeping gas containing up to 16% air, but sweeping for 3 hr with 32% air reduced the yield by 10%. Normal yields were obtained within 24 hr when sweeping with uncontaminated 70% N_2 and 30% CO_2 was resumed. Since contamination by any of the gases mentioned is likely to be very much less than the quantities tested, it was concluded that there would be little or no danger of their harming the fermentation.

External stripping of H_2S from fermenting sludge

When the apparatus was housed in the open laboratory (*c.* 20°) the sulphide yield obtained when both the fermenter and stripping column were kept at 30° was 22% less than the control (Table 4). That this effect was caused by cooling of the sludge, in those parts of the system other than the fermenter and column, was shown by the resumption of yields roughly equal to that of the control when the apparatus was transferred to a room kept at 30±2° (Table 4). Increasing the temperature of the stripping column to 40° increased the yields, both when the flow of sweeping gas was with or against the flow of sludge. At 40°, with gas flow against sludge circulation, there was an appreciable (9%) increase in yield compared with the control. On the other hand, there was a significant drop in yield with the stripping column at 45°, indicating that the flora responsible for sulphide production was harmed at this temperature. This effect agrees with that observed during experiments on the effect of temperature on sulphide production (Table 2). At 45°, and to a lesser extent at 40°, $CaCO_3$ was deposited on the walls of the stripping column, and this would

Table 4. *Comparison of S^{2-} yields from a fermenter with external stripping and from a control fermenter with internal stripping*

Temperature of stripping column (°C)	Yield‡ of S^{2-} (% w/v of raw sludge)	Yield‡ of S^{2-} from control (% w/v of raw sludge)
30*	0·72	0·90
30	0·88	0·90
30	0·74†	0·72
40*	0·82	0·82
40	0·63†	0·58
40	0·65§	0·78
45*	0·66	0·76

* Fermenter maintained at 30° in an open laboratory; all others in a room maintained at 30±2°. † Gas flow against sludge circulation. ‡Average results obtained for a 10-day RT over the period of steady conditions for each run. § Composition of sweeping gas 54% N_2 and 46% CO_2; all others 70% N_2 and 30% CO_2.

be troublesome in long term working. Such deposition was prevented by increasing the concentration of CO_2 in the sweeping gas from 30% to 46%, but this caused a considerable drop in sulphide yields.

This work was abandoned before all the experiments contemplated could be carried out, but the results obtained show that, provided certain cooling effects are avoided, the yields obtained are equal to (and, with countercurrent sweeping and with stripping column maintained at 40°, appreciably greater than) those obtained by sweeping through the whole volume of fermenting sludge. On a large scale the method would ease the problem of gas distribution and probably improve the efficiency of H_2S removal from the fermenting sludge.

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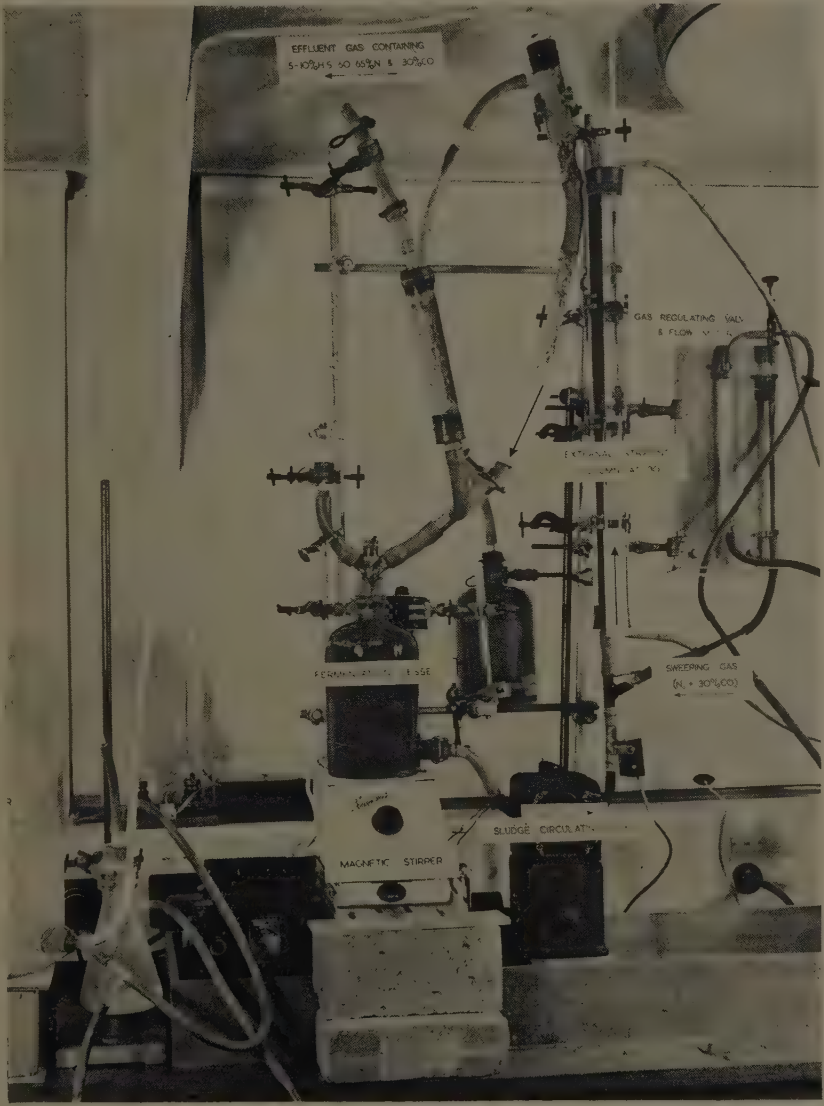
EXPLANATION OF PLATE

PLATE 1. Apparatus for the collection and measurement of sulphide from sewage sludges.

(*Received* 7 *January*, 1960)


EFFLUENT GAS CONTAINING
5-10% H S 50 65% N & 30% CO
GAS REGULATING VALV
& FLOW
SWEEPING GAS
(N + 30% CO)
SLUDGE CIRCULAT
MAGNETIC STIRRER

K. R. Butlin, Sylvia C. Selwyn and D. S. Wakerley — Microbial Sulphide Production from Sulphate-Enriched Sewage Sludge. Plate 1

Thomas, S. B., Druce, R. G. & Elson, K. (1960). *J. appl. Bact.* **23** (2), 169–184.

AN ECOLOGICAL STUDY OF THE COLI-AEROGENES BACTERIA OF SURFACE SOIL

By S. B. THOMAS, R. G. DRUCE and K. ELSON

National Agricultural Advisory Service, Trawscoed, Aberystwyth

SUMMARY: The coli-aerogenes organisms content, determined by plating on violet red-bile agar, of surface soil from a polluted site (heavily grazed pasture) often exceeded 10^4/g at 30° and 10^3/g at 37°. *Escherichia coli* I was present but in a small proportion only. High coli-aerogenes counts were much less frequent in unpolluted soil (enclosed Forestry Plantation), and *E. coli* I was relatively rare. The classification of 1,917 strains isolated from colonies on VRB agar at 30° and at 37° showed that *Klebsiella cloacae* was very much the dominant species in both sites when the plates were incubated at 30°, whereas *Citrobacter freundii* I and *K. cloacae* were co-dominant when incubation was at 37°. *E. coli* II constituted a relatively high proportion of the strains from both sites but, due to the very marked dominance of these three types, *E. coli* I was rarely isolated by the method employed, even from the polluted soil. The very high incidence of non-lactose-fermenters, particularly among the dominant types, was the outstanding characteristic of the strains isolated, even with incubation at 37°. Only 29% formed acid and gas in MacConkey's broth within 2 days at 37°, while 59% formed acid and gas within 5 days at 30° but failed to do so in 2 days at 37°. There was a much higher incidence of coli-aerogenes organisms in soil from the unpolluted site during summer than during winter, and this is attributed to possible multiplication of 37° negative strains of the more common types.

The relative incidence of different types of coli-aerogenes bacteria in soil freshly polluted with animal or human faeces and in apparently unpolluted soil fenced off from domestic animals has been the subject of several investigations during the past 50 years.

Escherichia coli I is considered to be exclusively faecal in origin, and it has been maintained that it does not multiply in soil but dies in a relatively short time. However, this organism may, under certain conditions, persist for many weeks in soil. Thus, Horrocks (1903) recovered *Escherichia* from sandy soil up to 9 weeks after adding suspensions of the organism, and more recently Cuthbert, Panes & Hill (1955) found that *E. coli* I which had been inoculated into limestone soil remained viable for 14 weeks. Thomas & Druce (unpublished data) studied the survival of this organism under natural conditions and found that it could persist in surface soil (pH 5·5–6·0) underlying cow manure in a pasture field for 20 weeks during May–September.

Representatives of the genera *Citrobacter* and *Klebsiella* may sometimes be present in considerable numbers in faeces, though they are generally very much outnumbered by *E. coli* I. But Buttiaux (1959) indicated that the use of antibiotics active against *E. coli* I (e.g. chloramphenicol and the tetracyclines) has greatly modified this situation so that *Klebsiella*, which is much more resistant to these antibiotics, may become so preponderant that the *Klebsiella*: *E. coli* I ratio may exceed 100:1. Irrespective of the origin of *Citrobacter* and *Klebsiella* strains, it is believed that they outlive *E. coli* I in all habitats other than the intestine, and thus become the predominant types in soil not subject to frequent and heavy faecal pollution from animals and birds.

The relative incidence in soil of members of the genera *Escherichia*, *Citrobacter* and *Klebsiella*, as determined in thirteen previous investigations, is summarized in Table 1. In ten of these investigations primary isolation of the coli-aerogenes strains was done at 37°, so that the presence of 37° negative strains, which we now know constitute the greater portion of the coli-aerogenes microflora of soil, would only rarely have been detected.

Table 1. *The incidence of different types of coli-aerogenes bacteria found in soil by previous investigators*

Investigators	Soil pollution	No. of strains	Incidence (%) of:			
			Escherichia	*Citrobacter*	*Klebsiella*	Irregular
Johnson & Levine (1917)	Absent (fallow)	177	19·6	80·4	0·0	0·0
Chen & Rettger (1920)	Absent	467*	4·3	95·7	0·0	0·0
Koser (1924)	Absent	72*	2·8	31·9	65·3	0·0
Koser (1926)	Light	104*	23·1	7·7	67·3	1·8
	Heavy	33*	63·6	3·0	33·3	0·0
Ragavachari (1926)	Absent	518	5·0	1·5	93·5	0·0
Burke-Gaffney (1932)	Absent	40†	0·0	0·0	100	0·0
	Remote	24†	4	29	67	0·0
	Recent	36†	81	8	11	0·0
	Remote	24‡	29	58	4	9
	Recent	120‡	80	6	3	11
Bardsley (1934)	Absent	152	31·0	66·4	2·6	0·0
Kline (1935)	Present (pasture)	30	76·6	16·7	6·6	0·0
Griffin & Stuart (1940)	Absent	174	2·3	4·6	93·1	0·0
	Remote	213	8·9	9·9	81·2	0·0
	Recent	203	34·7	3·4	61·9	0·0
Frank & Skinner (1941)	Absent	356	21·0	34·3	44·7	0·0
Taylor (1951)	Absent	85**	8·2	61·2	30·6	0·0
Fraser, Reid & Malcolm (1956)	Mainly present	82§	49·0	34·0	16·0	1·0
		No. of samples	Samples (%) containing:			
			Escherichia	*Citrobacter*	*Klebsiella*	None
Randall (1956)	Present (grasslands)	110	18·1	71·8	12·7	11·8
	Absent (protected grasslands)	98	10·2	64·2	8·1	23·4

* Isolated at 30°. ** Including 37° negative strains. † Soil from Dar-es-Salaam. ‡ Soil from Dublin. § Isolated at 25° and 37°.

In unpolluted soil *Klebsiella* was the dominant type in 5 investigations and *Citrobacter* in 3. *Escherichia* generally constituted less than 10% of the strains isolated from unpolluted sites, although it formed 21% and 31% in two investigations (Frank & Skinner, 1941; Bardsley, 1934). In sharp contrast *Escherichia* often constituted 60–80% of the strains isolated from polluted soil, with *Citrobacter* and *Klebsiella* relatively infrequent.

The present investigation of the incidence of the different types of coli-aerogenes bacteria in the surface soil of a heavily grazed pasture (polluted site) and an ungrazed upland Forestry Commission enclosure (relatively unpolluted site) was undertaken with the object of comparing the results obtained with primary isolation on a solid selective medium at 30° with those at 37°.

MATERIALS AND METHODS

Sampling and enumeration. Two series of soil samples were examined in the period October, 1956–December, 1958. The unpolluted series comprised 82 samples taken from the surface soil (pH 3·5–6·0) of a Forestry Commission Plantation which had been enclosed about 3 years previously and planted with conifers. It had at one time a high rabbit population, but this had been completely exterminated by myxomatosis before the sampling began. The part of the plantation sampled (3 acres) lies between 600 and 800 ft above sea level and it adjoins the Trawscoed Experimental Husbandry Farm. The polluted series of 82 samples was taken from the surface soil (pH 5·0–7·0) of a heavily grazed, permanent pasture field (6 acres) adjoining the homestead of the same farm. It is 200–250 ft above sea level, three-quarters of a mile from the Forestry Plantation, and is grazed at intervals throughout the year by about 120 milking cows.

Representative samples of surface soil from both sites were taken on the same day at approximately weekly intervals. Bores 1 in. deep were taken from 25 random and equally spaced points over half of each site; the left and right halves were sampled during alternate weeks. The composite sample weighed about 100 g.

Within 2 hr of sampling, each composite sample was thoroughly mixed on a sterile glass sheet and 10 g weighed into a sterile 750 ml bottle, to which 90 ml of quarter-strength Ringer's solution were added; the bottle was then closed with a sterile rubber stopper and placed on a shaking machine for 30 min. From this 10^{-1} dilution serial tenfold dilutions (10^{-2}–10^{-4}) were prepared in quarter-strength Ringer's solution and duplicate sets of each dilution were plated on violet red-bile agar (Druce *et al.* 1957). One set of plates was incubated for 48 hr at 30° and the other for 48 hr at 37°. One ml of the 10^{-1} soil suspension was also 'plated' on violet red-bile agar (VRB) in a 4 oz 'medical flat' bottle which was incubated in a water bath for 48 hr at $44 \pm 0{\cdot}5°$ to determine the *E. coli* I content.

The following additional primary inoculations were made to facilitate the detection of small numbers (<10/g) of coli-aerogenes organisms: (a) 1 g amounts into duplicate tubes of MacConkey's broth, one set being incubated for 5 days at 30° and the other for 48 hr at 37°, and (b) 1 g into a tube of brilliant green-bile broth, which was incubated in a water bath for 48 hr at $44 \pm 0{\cdot}5°$. When positive, the presence of *E. coli* I was confirmed by streaking a loopful on a dried plate of eosin-methylene blue agar, incubating for 24 hr at 37°, and then testing representative colonies by the 44° indole test.

Isolation and differential tests. The numbers of purple-red colonies on VRB agar incubated at 30° and at 37° were recorded as the presumptive coli-aerogenes contents of soil and the colony counts at 44° were recorded as the presumptive *E. coli* I contents/g.

Six colonies picked from the VRB plates incubated at 30° and 6 from those incubated at 37° were inoculated into peptone water incubated at 30°. Whenever possible 2 colonies were picked from each of the plates of the three highest dilutions showing characteristic coli-aerogenes colonies (10^{-1}–10^{-3} or 10^{-2}–10^{-4}). After purification by streaking, a 24 hr peptone water culture provided the inoculum for the differential tests, which were carried out according to the technique recommended in Report (1949), an incubation temperature of 30° being used except as indicated below. These tests included indole production, the methyl red test, the Voges-Proskauer test and citrate utilization (the IMViC tests), plus the Gram reaction, lactose fermentation, the reaction in MacConkey's broth at 37° and 44° and gelatin liquefaction within 21 days at 22°.

Non-lactose-fermenters are defined as: 37° negative when no gas was formed in MacConkey's broth in 48 hr at this temperature; anaerogenic when acid but no gas was formed in lactose broth in 5 days at 30°; and paracolon when neither acid nor gas was formed in 5 days at 30°. The nomenclature proposed in Report (1956) was applied to the strains studied.

RESULTS

The incidence of different categories of coli-aerogenes bacteria

The results are shown in Table 2. All the soil samples from both the sites contained coli-aerogenes bacteria in 10^{-1} g amounts as judged by tests at 30°, i.e. they yielded characteristic coli-aerogenes colonies on VRB agar and the organisms formed acid and gas in lactose within 5 days at 30°. One g amounts of all samples also gave acid and gas in MacConkey's broth in 5 days at 30°.

Table 2. *The incidence of different categories of coli-aerogenes bacteria in* 82 *samples of surface soil from grazed pasture* (G) *and* 82 *samples from an ungrazed plantation* (UG)

Grouping of samples	No. of samples from	
	G	UG
Isolation from colonies on VRB agar		
Positive in 10^{-1} g for strains which formed characteristic colonies in 48 hr at 30°	82	82
Positive for strains which formed acid and gas from lactose in 5 days at 30°	82	82
Positive in 10^{-1} g for strains which formed characteristic colonies in 48 hr at 37°	81	81
Positive for strains which formed acid and gas in MacConkey's broth in 48 hr at 37°	66	50
Positive in 10^{-1} g for strains which formed characteristic colonies in 48 hr at 44°	38	10
Positive for strains which formed gas in brilliant green-bile broth in 48 hr at 44°	30	2
Isolation via selective broth		
Positive in 1 g for strains which formed acid and gas in MacConkey's broth in 5 days at 30°	82	82
Positive in 1 g for strains which formed acid and gas in MacConkey's broth in 48 hr at 37°	81	81
Positive in 1 g for strains which formed gas in brilliant green-bile broth in 48 hr at 44°	61	5

While 81 samples from each site gave characteristic coli-aerogenes colonies on VRB agar in 48 hr at 37°, a third of the isolates from the polluted site and half of those from the unpolluted site did not ferment lactose in 48 hr at 37°, i.e. they were 37° negative.

E. coli I was present in a fairly high proportion of the samples from the polluted site. Thus 61 samples (74%) contained *E. coli* I in 1 g amounts as shown by formation of gas at 44° in brilliant green-bile broth. Only 38 samples (46%) gave characteristic purple-red colonies at 44° on VRB agar inoculated with 10^{-1} g, and the presence of *E. coli* I was confirmed in 30 of these. On the other hand, *E. coli* I was relatively rare in samples from the unpolluted soil. Only 5 samples (6%) gave positive results when 1 g amounts were inoculated into brilliant green-bile broth at 44°. Ten (12%) of the 10^{-1} g amounts produced purple-red colonies at 44°, but the presence of *E. coli* I was confirmed in only 2 of these.

Coli-aerogenes colony counts on VRB agar

The counts obtained are given in Table 3. Soil from the polluted site often contained large numbers of coli-aerogenes bacteria; colony counts on VRB agar in excess of 10^4/g were recorded for 73% of the samples at 30° and for 50% at 37°. None gave a colony count of this magnitude at 44°, but presumptive *E. coli* I was present in small numbers (but above 10/g) in 46%. High coli-aerogenes colony counts ($>10^4$/g) were much less frequent in the soil from the unpolluted site, only 28% and 12% of the samples giving counts of this order at 30° and 37° respectively. Nevertheless, all the samples contained some coli-aerogenes organisms. Thus counts exceeding 10^2/g were recorded for all samples at 30° and for 95% at 37°. Relatively few samples contained *E. coli* I, only 12% giving colony counts larger than 10/g at 44°.

Table 3. *A comparison of the coli-aerogenes counts, on VRB agar at three temperatures, of 82 samples each of soil from a polluted and an unpolluted site*

Temp. of incubation of plates	Type of soil*	No. of samples with colony counts†/g in the ranges:					
		<10	10^1–10^2	$>10^2$–10^3	$>10^3$–10^4	$>10^4$–10^5	$>10^5$–10^6
30°	P	0	0	1	21	49	11
	UP	0	0	8	51	19	4
37°	P	1	0	7	33	35	6
	UP	1	2	24	45	9	1
44°	P	44	28	10	0	0	0
	UP	72	6	4	0	0	0

* P, polluted site (heavily grazed pasture); UP, unpolluted site (Forestry Commission enclosure). † On VRB agar incubated for 48 hr at the indicated temperature.

The classification of organisms isolated at 30°

The incidence of different types among 960 cultures isolated at 30° is given in Table 4. *Klebsiella* and *Citrobacter* formed a very high proportion of the coli-aerogenes microflora, with the consequence that no strains of *E. coli* I were isolated, though it has already been shown that this organism was present in small numbers, even in the unpolluted soil, where possibly 6% of the samples contained it. Strains of *E. coli* II, however, constituted about 5% of the cultures from both sites, and it is significant that most of them were 37° negative.

K. cloacae was very much the dominant species in the soil from both sites, forming 75% of the strains from the polluted site and 62% of those from the unpolluted site. The preponderance of *K. cloacae* among the coli-aerogenes organisms in soil from fallow and cropped experimental arable plots has already been reported by Johnson & Levine (1917), and the very high incidence of *K. cloacae* in strains isolated at 30° is similar to that reported for grass and cereal crops (Thomas & McQuillin, 1952; Thomas & Hobson, 1955). In contrast, *K. aerogenes* I was of relatively infrequent occurrence in soil.

Table 4. *The types of coli-aerogenes bacteria isolated at* 30° *from surface soil*

Organism and type	Cultures from site:					
	Polluted			Unpolluted		
	No.		%	No.		%
Escherichia coli I	0		0·0	0		0·0
E. coli II	2	25	5·2	1	28	5·9
37° negative	15			21		
37° negative, gelatin positive	2			2		
anaerogenic	2			3		
anaerogenic, gelatin positive	4			1		
Citrobacter freundii I	17	49	10·1	18	126	26·5
gelatin positive	3			2		
37° negative	18			59		
37° negative, gelatin positive	4			29		
anaerogenic	3			14		
anaerogenic, gelatin positive	0			2		
paracolon	4			2		
Cit. freundii II	4	24	4·9	2	10	2·1
44° positive	2			0		
gelatin positive	2			1		
37° negative	4			5		
37° negative, gelatin positive	5			1		
anaerogenic	1			1		
anaerogenic, gelatin positive	3			0		
anaerogenic, citrate negative	2			0		
paracolon, gelatin positive	1			0		
Klebsiella aerogenes I	12	22	4·5	6	17	3·6
37° negative	6			4		
anaerogenic	3			5		
paracolon	1			2		
K. cloacae	99	364	75·1	57	294	61·9
indole positive	1			2		
37° negative	242			188		
37° negative, indole positive	5			12		
anaerogenic	11			19		
anaerogenic, indole positive	2			0		
paracolon	4			16		
K. aerogenes II	0	1	0·2	0	0	0·0
37° negative	1			0		
Total strains	485			475		

Cit. freundii I was the next most abundant species isolated at 30°, particularly from unpolluted soil, where it constituted 26% of the strains compared with 10% in polluted soil. A high incidence of this organism in unpolluted soil has previously been reported by Bardsley (1934), Taylor (1951) and Randall (1956). It is of interest to note that a higher incidence of *Cit. freundii* I in unpolluted as compared with polluted water has also been reported; Thomas, Hobson & Druce (1959) found that it constituted 31% of 216 coli-aerogenes strains isolated at 30° from unpolluted farm water supplies, but only 9% of 120 strains isolated from polluted water.

Table 5. *The types of coli-aerogenes bacteria isolated at 37° from surface soil*

Organism and type	Cultures from site:					
	Polluted			Unpolluted		
	No.		%	No.		%
Escherichia coli I	10	12	2·5	0	1	0·2
44° negative	2			1		
E. coli II	6	37	7·6	9	63	13·4
37° negative	17			41		
37° negative, gelatin positive	6			0		
anaerogenic	1			11		
anaerogenic, gelatin positive	6			0		
paracolon	1			2		
Citrobacter freundii I	79	157	32·4	55	161	34·1
gelatin positive	0			3		
37° negative	62			77		
37° negative, gelatin positive	6			8		
anaerogenic	7			15		
anaerogenic, gelatin positive	1			0		
paracolon	2			3		
Cit. freundii II	6	35	7·2	17	45	9·5
44° positive	2			0		
gelatin positive	1			0		
37° negative	12			24		
37° negative, gelatin positive	3			0		
anaerogenic	4			3		
anaerogenic, gelatin positive	4			0		
anaerogenic, citrate negative	1			1		
paracolon	2			0		
Klebsiella aerogenes I	28	49	10·1	5	40	8·5
37° negative	16			14		
anaerogenic	2			8		
paracolon	3			13		
K. cloacae	51	190	39·2	47	153	32·4
indole positive	1			0		
37° negative	129			78		
37° negative, indole positive	1			1		
anaerogenic	5			16		
anaerogenic, indole positive	3			0		
paracolon	0			11		
K. aerogenes II	2	5	1·0	0	9	1·9
37° negative	1			2		
anaerogenic	1			5		
paracolon	1			2		
Total strains	485			472		

Apart from the higher incidence of *Cit. freundii* I and the somewhat lower incidence of *K. cloacae* in the unpolluted soil than in the polluted soil, there was no marked difference between the coli-aerogenes microfloras isolated at 30° from the two sites.

The classification of organisms isolated at 37°

Cit. freundii I and *K. cloacae* were co-dominant among the strains isolated from both sites at 37° (Table 5). The former was much more common among the strains isolated at 37° than among those isolated at 30°. On the other hand, *K. cloacae* was less frequently isolated at 37°; at this temperature it constituted only about a third of the strains, compared with 75% and 62% respectively of the strains from polluted and unpolluted soil when the isolation temperature was 30°.

K. aerogenes I formed a somewhat higher proportion with isolation at 37° than when the temperature was 30°, but even so it constituted only 10% of all the strains. A similar low proportion of this type was recorded, with isolation at 37°, in farm water supplies (Thomas, Hobson & Druce, 1959).

A few strains of *E. coli* I were isolated from the polluted soil and one 44° negative strain from the unpolluted soil: but *E. coli* II was relatively common particularly in unpolluted soil. There have been previous references to the probability of *E. coli* II being a characteristic soil type (Taylor, 1949: Taylor, 1951).

Cit. freundii II formed nearly 10% of the strains, and it has been suggested that its presence in polluted water, where it is not uncommon, is probably due to surface washings from soil (Thomas, Hobson & Druce, 1959).

A direct comparison of the incidence of the seven types isolated at 30° and at 37° from both sites is given in Table 6. Though there were appreciable differences between the incidences of different types in the polluted and the unpolluted soils, they were not nearly so marked as those between the strains isolated at 30° and 37°.

Table 6. *A comparison of the types of coli-aerogenes bacteria isolated at 30° and 37° from polluted sites* (P) *and unpolluted sites* (UP)

Organism and type	Cultures (%) isolated at:			
	30°, from site		37°, from site	
	P	UP	P	UP
E. coli I	0·0	0·0	2·5	0·2
E. coli II	5·2	5·9	7·6	13·4
Cit. freundii I	10·1	26·5	32·4	34·1
Cit. freundii II	4·9	2·1	7·2	9·5
K. aerogenes I	4·5	3·6	10·1	8·5
K. cloacae	75·1	61·9	39·2	32·4
K. aerogenes II	0·2	0·0	1·0	1·9

The classification of cultures forming or not forming gas from lactose

An outstanding characteristic of the coli-aerogenes strains derived from both the soils tested was the very high incidence of non-lactose-fermenters. The detailed classifications given in Tables 4 and 5 show that their incidence was very similar for all types derived from both sites. The data have therefore been combined, and in Table 7 the percentages of the 37° negative, anaerogenic and paracolon strains together of each type isolated at 30° are compared with those isolated at 37°. The finding that as many as 76% of the 960 strains isolated at 30° did not produce acid and gas in MacConkey's broth within 2 days at 37° was not unexpected, as previous investigations had shown that 37° negative strains constituted a high proportion of the cultures isolated at 30° from water, ungrazed herbage and cereal crops, where they formed 38%, 59% and 71% respectively (Thomas, Hobson & Druce, 1959; Thomas & McQuillin, 1952; Thomas & Hobson, 1955). The presence of a high proportion (66%) of such 37° negative strains among the 957 cultures isolated from coli-aerogenes colonies on VRB agar incubated at 37° was however unexpected, although a much smaller incidence (6%) had been recorded under similar circumstances for farm water supplies (Thomas, Hobson & Druce, 1959).

Apart from *E. coli* I, of which there were no strains which failed to form gas at 37°, all the types included a high proportion of such strains irrespective of the isolation temperature. Moreover, the incidence was highest among the dominant types, *K. cloacae*, *Cit. freundii* I and *E. coli* II.

Table 7. *The effect of isolation temperature on the incidence of* 37° *negative, anaerogenic and paracolon strains* (*combined data from both sites*)

Type	Isolation at 30°			Isolation at 37°		
	Total no.	37° negative, anaerogenic and paracolon		Total no.	37° negative, anaerogenic and paracolon	
		No.	%		No.	%
E. coli I	0	0	0·0	13	0	0·0
E. coli II	53	50	94·3	100	85	85·0
Cit. freundii I	175	135	77·1	318	181	57·0
Cit. freundii II	34	23	67·6	80	54	67·5
K. aerogenes I	39	21	53·9	89	56	62·9
K. cloacae	658	499	75·8	343	244	71·1
K. aerogenes II	1	1	100·0	14	12	85·7
Totals	960	729	75·9	957	632	66·0

The distribution of the 'fermentation groups'

As there were no marked differences due to the temperature of isolation in the incidence of strains failing to produce gas at 37°, the distribution of the three 'fermentation groups' (37° positive, 37° negative and anaerogenic plus paracolon types) among different types was calculated for all the 1,917 strains examined, with the result shown in Table 8.

Only 29% of all the strains produced acid and gas in MacConkey's broth within 2 days at 37°, 59% were 37° negative but gave acid and gas in 5 days at 30° and 12% were either anaerogenic or of the paracolon type. Of the 1,361 strains which failed to form gas at 37°, 1,121 (82%) formed acid and gas within 5 days at 30°.

A high proportion of the strains of both *Cit. freundii* I and *K. cloacae* were 37° negative, but most of these gave gas in 5 days at 30°, anaerogenic and paracolon strains being relatively infrequent.

E. coli II was characterized by a remarkably low proportion of 37° positive strains, a high incidence of 37° negative strains and a moderate number of anaerogenic and paracolon strains. *K. aerogenes* II had the highest proportion of anaerogenic and paracolon strains.

Table 8. *The distribution of 'fermentation groups' among different types of coli-aerogenes bacteria isolated at 30° and at 37° from surface soil from polluted and unpolluted sites*

Organism and type	Group A*		Group B*		Group C*		Total no. of type
	No.	%	No.	%	No.	%	
E. coli I	13	100·0	0	0·0	0	0·0	13
E. coli II	18	11·7	104	68·0	31	20·3	153
Cit. freundii I	177	35·9	263	53·3	53	10·8	493
Cit. freundii II	37	32·4	54	47·4	23	20·2	114
K. aerogenes I	51	39·8	40	31·2	37	28·9	128
K. cloacae	258	25·8	656	65·5	87	8·7	1,001
K. aerogenes II	2	13·3	4	26·7	9	60·0	15
Totals	556	29·0	1,121	58·5	240	12·5	1,917

* Fermentation group: A, 37° positive; B, 37° negative; C, anaerogenic plus paracolon strains.

Seasonal incidence

A comparison of the colony counts during winter (November–February) and summer (May–August) is given in Table 9. Soil from the polluted site showed no significant differences in its coli-aerogenes organisms content in winter and summer. With isolation at 30° counts exceeding 10^4/g were recorded for 79% of the winter samples and 73% of the summer samples, and using 37° the respective proportions for winter and summer were 47% and 50%. This was to be expected, the pasture sampled being the paddock adjoining the farmstead and as the dairy cows were exercised there in the winter it was subject to faecal contamination throughout the year.

Table 9. *The seasonal incidence of coli-aerogenes organisms in polluted and unpolluted soil*

Type of soil*	Season†	Temp. of isoln.	No. of samples with colony counts‡/g in the ranges						Total samples
			<10	10^1–10^2	$>10^2$–10^3	$>10^3$–10^4	$>10^4$–10^5	$>10^5$	
P	Winter	30°	0	0	0	8	21	5	34
		37°	0	0	2	16	12	4	34
	Summer	30°	0	0	0	6	15	1	22
		37°	1	0	2	8	10	1	22
UP	Winter	30°	0	0	6	28	0	0	34
		37°	0	2	13	19	0	0	34
	Summer	30°	0	0	0	9	11	2	22
		37°	1	0	6	8	6	1	22

* P, polluted site; UP, unpolluted site. † Winter, Nov.–Feb.; summer, May–Aug. ‡ On VRB agar, incubated for 48 hr at indicated temperature.

The coli-aerogenes organisms content of soils from the unpolluted site was very much higher in summer than in winter. None of the winter samples contained more than 10^4 coli-aerogenes bacteria/g with isolation at either temperature, whereas 59% of the counts at 30° and 32% of those at 37° exceeded that level in summer. There was no evidence that the unpolluted site was more subject to faecal contamination in the summer than in the winter, and the increase can only be attributed to multiplication of probably the 37° negative strains of the three dominant types during warm weather. The soil on this site is blanketed with a layer of decaying vegetation (mainly Yorkshire Fog, bent, and fescue together with bracken and moss) especially during late autumn and winter. This may, by summer, provide organic material suitable for the multiplication of these bacteria.

A comparison of the seasonal distribution of the different types isolated from the unpolluted site showed that 37° negative types were rather more frequent in summer; 87% of the 253 strains isolated at 30° and 37° in summer were 37° negative, but only 72% of the 422 winter isolates were of that type. *Cit. freundii* I formed a higher proportion of the strains isolated at 30° in summer than in winter (34% against 20%), while *K. cloacae* showed a corresponding smaller incidence in summer (57% against 65%). The only significant seasonal difference among the 37° isolates was a markedly lower frequency of *E. coli* II in summer (4% against 15%).

Contrary to expectation, no significant differences were observed in the coli-aerogenes contents of soil from either site during rainy periods and dry or frozen periods in winter or summer.

Aberrant strains

The occurrence of gelatin liquefying strains of *Citrobacter* (the *Arizona* group of Edwards, West & Bruner, 1947) was nearly as striking as that found in water by Thomas, Hobson & Druce (1959). Of the 493 strains of *Cit. freundii* I isolated (Tables 4 and 5) 12% liquefied gelatin within 21 days at 22°; the proportion was 19% among the 295 strains from farm water supplies. Again, 18% of the 114 strains of *Cit. freundii* II isolated hydrolyzed gelatin; the proportion among the 98 strains from water was 10%.

The incidence of 44° positive strains of *Citrobacter* and *Klebsiella* was low, much lower than in water. Only 4 such strains, which were of *Cit. freundii* II and from the polluted site, were encountered, whereas 35 of the 934 *Citrobacter* and *Klebsiella* strains from water were 44° positive. Their presence appears to be associated with faecal pollution, as they were also most prevalent in contaminated water containing appreciable numbers of *E. coli* I.

The 19 paracolon strains of *K. aerogenes* I, 15 of which were isolated from the unpolluted site, probably represent the *Hafnia* group of Moeller (1954). They constituted 15% of the 128 strains of *K. aerogenes* I isolated in the present work compared with 7% of the 212 strains of this type previously isolated from water.

There were 22 (3%) indole positive strains among the 658 cultures of *K. cloacae*. Nonmotile, indole positive and generally gelatin liquefying strains of *Klebsiella* have been assigned to the *Oxytoca* group by Hugh (1959), and organisms of this kind have previously been found in soil, human and bovine faeces, milk and cheese. The present cultures were not examined for motility.

The relative incidence of different types in soil, grass and water

A comparison of the incidence of the three most frequently occurring types of coli-aerogenes bacteria in soil, grass and water is given in Table 10, where the terms dominant, frequent and occasional are used in their ecological connotation (Tansley, 1939) and the figures in brackets after each type are its percentage frequency. In all cases the strains were isolated at 30°, and the comparisons are for soil examined during the present investigation, grass examined by Thomas & McQuillin (1952) and farm water supplies examined by Thomas, Hobson & Druce (1959). The soil and grass samples were taken from the same polluted and unpolluted sites, while water supplies giving presumptive *E. coli* I counts exceeding 250/100 ml at 44° have been treated as polluted sources and waters giving negative tests for *E. coli* I have been classed as unpolluted sources.

Table 10. *The most common types of coli-aerogenes bacteria in different habitats, as judged by isolation at 30°, and their comparative incidence*

Source and no. of strains isolated		Types* classed ecologically as:		
		Dominant	Frequent	Occasional
Polluted				
Soil	485	*K. cloacae* (75)	*Cit. freundii* I (10)	*E. coli* II (5)
Grass	139	*K. cloacae* (51)	*K. aerogenes* I (18)	*Cit. freundii* I (14)
Water	120	*E. coli* I (42)	*K. cloacae* (16)	*K. aerogenes* I (13)
Unpolluted				
Soil	475	*K. cloacae* (62)	*Cit. freundii* I (26)	*E. coli* II (6)
Grass	110	*K. cloacae* (66)	*Cit. freundii* I (19)	*K. aerogenes* I (9)
Water	216	*K. cloacae* (35)	*Cit. freundii* I (31)	*K. aerogenes* I (18)

* The numbers in brackets indicate the percentages of strains isolated.

It will be seen that the distribution is characterized by the dominance of *K. cloacae* in all habitats except polluted water, where *E. coli* I was dominant, and the frequent occurrence of *Cit. freundii* I particularly among strains isolated from unpolluted habitats. While *K. aerogenes* I constituted an appreciable proportion of the strains from grass and water, it was less common than *E. coli* II in soil from both the polluted and unpolluted sites.

The dominance of *K. cloacae* in soil has been attributed to its persistence and longer survival than other types (Prescott, Winslow & McCrady, 1946). Its somewhat higher incidence in polluted as compared with unpolluted soil in the present investigation does not support this contention. On the other hand, the dominance of the essentially motile *K. cloacae* and *E. coli* I in the natural habitats surveyed above may be significant, as this character may result in their wider distribution in soil moisture and surface water than the nonmotile *K. aerogenes* can achieve.

DISCUSSION

Incubation for 20–24 hr at 30° has been recommended for the determination of the coli-aerogenes colony count on VRB agar of milk and milk products (Druce *et al.* 1957), but incubation for 48 hr at both 30° and 37° was necessary with VRB agar inoculated with soil suspensions, as the coli-aerogenes colonies developed more slowly. Nevertheless, most of the colonies picked from the 48 hr plates were typical coli-aerogenes organisms: 91% of the 494 colonies from the 30° plates and 91% of the 487 colonies from the 37° plates inoculated with suspensions from the polluted soil formed acid and gas from lactose within 5 days at 30°, though the proportions were somewhat lower among the colonies derived from unpolluted soil, 86% of the 491 colonies from the 30° plates and 81% of the 500 colonies from the 37° plates being confirmed. These results compare favourably with those obtained with milk, where 82% of the 1,406 deep red colonies over 0·5 mm in diameter were proved to be coli-aerogenes organisms (Druce *et al.* 1957).

Colonies of *Serratia* could not always be distinguished from those of coli-aerogenes bacteria, and 42 of the colonies picked from the VRB plates inoculated with soil suspensions were found, after purification and further cultural examination, to be *Serratia* spp. As many as 37 (88%) of these formed acid and gas from lactose within 5 days at 30°. In contrast, only 21 (19%) of the 110 strains of *Serratia*, mainly derived from dairy products and water, studied by Thomas & Lewis (1957) formed acid and gas from lactose in 5 days at 30°, and it would appear that unpolluted soil forms a natural habitat of aerogenic strains of this genus, because all except 3 of the 42 strains were isolated from soil from the unpolluted site. The temperature of incubation of the VRB plates did not appear to have any marked selective effect, as 24 of the *Serratia* strains were picked from the 37° plates and 18 from the 30° plates.

Incubation of VRB agar for 48 hr at 44° proved satisfactory for the detection of *E. coli* I; 77 (80%) of the 96 colonies examined formed indole in 24 hr at 44° and gas in brilliant green-bile broth in 48 hr at 44°.

A comparison of the prevalence in soil of the different types of coli-aerogenes organisms isolated during the present investigation with the data obtained during recent surveys in Britain by Bardsley (1934), Taylor (1951) and Randall (1956) is of interest. Bardsley took samples from just beneath the surface, after the upper layer of soil had been scraped away. The soil was peaty and very acid (lowest pH value, 4·0) and the vegetation was mainly of Ericaceous plants commonly found on well drained peaty ground in the Pennines. A 15 g sample was ground in sterile water and the volume made up to 150 ml. Primary isolation was made by inoculating dilutions of this soil suspension into MacConkey's broth and incubating for 48 hr at 37°. No coli-aerogenes bacteria were detected in 76% of the 86 samples, *Citrobacter* was found in 20%, *Escherichia* in 8% and *Klebsiella* in 2%. Of the 152 strains isolated from the unpolluted soil by this method, 66% were *Citrobacter*, 31% *Escherichia* and 3% *Klebsiella*. Taylor (1951) scraped away the surface soil ($\frac{1}{4}$–$\frac{1}{2}$ in.) and sampled the underlying $2\frac{1}{2}$–3 in. The soil samples were derived from several widely separated localities and were sometimes many days old when examined. With primary isolation in MacConkey's broth at 37°, coli-aerogenes organisms were found in low numbers in 73% of the samples examined; only 33% gave confirmed counts exceeding 100/g of soil. The technique was designed to detect organisms of sanitary significance only, and it is not surprising that Taylor concluded that *E. coli* I was the most prevalent type, whatever the location of the soil or its type of vegetation. Nevertheless, his results for 85 strains from the Broadbalk field at Rothamsted Experimental

Station, when classified irrespective of whether they formed gas in MacConkey's broth at 37° or not, showed that *Citrobacter* constituted 61%, *Klebsiella* 31% and *Escherichia* only 8%. Randall (1956) used MacConkey's agar in roll tubes incubated for up to 3 days at 37° for the primary isolation of coli-aerogenes cultures from soil from gardens, woods and protected grasslands alongside enclosed reservoirs. About 1 g of surface soil was collected in a 1 oz screwcapped bottle and shaken by hand with twice its weight of Ringer's solution. Relatively few samples of soil from sites open to animal pollution contained more than 2,000 coli-aerogenes organisms/g, but *E. coli* I occurred in most of the samples from these sites, usually in low numbers. *Citrobacter freundii* I was the dominant organism in many of the soil samples from protected grassland, but it rarely exceeded 2,000/g and in most samples it was found only in low numbers (2–10/g). Randall maintained that these numbers were not of the order to be expected of bacteria living in their natural environment and therefore concluded that *Citrobacter* does not actively establish itself in soil. In the present investigation various factors combined to give a much higher and probably more accurate estimate of the coli-aerogenes content of soil than could have been obtained from an investigation concentrated on the types of sanitary significance in which primary isolation at 37° only was used. These factors were: the sampling of the more heavily infected 1 in. of surface soil; the thorough mechanical shaking of a relatively large amount of soil, selected from an original sample of about 100 g in a large volume of Ringer's solution; and primary isolation of representative cultures within 2 hr of sampling by duplicate plating on a solid selective medium with incubation at both 30° and 37°. Very few of the samples examined by Taylor (1951) and Randall (1956) had coli-aerogenes counts in excess of 10^3/g, whereas nearly all the samples in the present investigation had counts of this magnitude and some, even from the unpolluted site, contained more than 10^5/g.

Every effort was made to make the investigation a truly ecological survey and therefore little attention has been given to the sanitary significance of the findings. Nevertheless, it is apparent that the method employed in determining the incidence of different types of coli-aerogenes organisms favoured the selection of the types present in largest numbers, and it is believed that the use of a solid selective agar medium is better than the broth dilution method for this purpose.

The inclusion of 37° negative strains, even when VRB plates were incubated at 37°, is particularly valuable; strains which fail to form gas from lactose in 48 hr at 37° do form acid and thus appear as characteristic purple-red colonies on the plates.

The general ecological pattern shown by the results of the present investigation, irrespective of whether primary isolation was at 30° or 37°, was the overwhelming predominance of *K. cloacae*, the frequent occurrence of *Cit. freundii* I, particularly in unpolluted soil, and the fairly regular presence of *E. coli* II; *E. coli* I, however, was relatively infrequently encountered. The 1,650 strains of these three commonly occurring types constituted 86% of the 1,917 strains classified, and it is highly significant that 72% of them did not form acid and gas from lactose in 48 hr at 37°.

Coli-aerogenes organisms constituted less than 0·1% of the bacterial content of the soil, as determined by a colony count on soil extract agar incubated for 14 days at 26°, but their constant presence in appreciable numbers (10^3–10^5/g) in unpolluted soil indicates that they are widely distributed in nature. This does not necessarily refute the view that certain types of coli-aerogenes bacteria in soil and vegetation are originally derived from faecal contamination by animals and birds. Nevertheless, it is probable that the 37° negative strains of the dominant types (*K. cloacae*, *Cit. freundii* I and *E. coli* II) multiply in soil under certain circumstances. Indirect evidence of this was provided by the significantly larger coli-aerogenes colony counts at both 30° and 37° given by soil from the unpolluted site in the summer as compared with the winter. But there was no marked seasonal difference in the coli-aerogenes content of the soil from the polluted site; this could be accounted for by the heavy grazing and consequent high faecal contamination which occurred in winter as well as in summer.

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THE RAPID TENTATIVE RECOGNITION OF PSYCHROTROPHIC TYPES AMONG ENTEROBACTERIACEAE ISOLATED FROM FOODS

By D. A. A. MOSSEL and H. ZWART

Central Institute for Nutrition and Food Research T.N.O.,
Utrecht, The Netherlands

SUMMARY: Enterobacteriaceae which can grow at the temperatures prevailing in refrigerated food stores ('psychrotrophic' Enterobacteriaceae) can be distinguished from strains which cannot develop below 10° because the latter generally grow quickly on nutrient agar at 43 ± 0·1° whereas the former do not. By this test inadequately refrigerated foods can be differentiated from those kept properly refrigerated.

It has long been recognized that among the many orthodox types of Enterobacteriaceae, strains capable of developing under conditions of commercial and domestic refrigeration, *i.e.* below 10°, may occur (Ayers & Clemmer, 1918). It is suggested that such bacteria should be called psychrotrophic variants, from *psychros*, cold and *trephein*, to nourish upon or to develop, as in eutrophic, dystrophic, hypertrophic etc. (cf. Eddy, 1960). Such psychrotrophic bacteria are not necessarily psychrophilic, in the sense suggested by Ingraham (1958) and Ingraham & Bailey (1959).

Since the discovery of psychrotrophic types of Enterobacteriaceae they have been isolated from various foods such as milk (Sherman & Wing, 1933; Dahlberg, 1946; Greene & Jezeski, 1954; Marth & Frazier, 1957; Parry & Tee, 1958; Panes & Thomas, 1959), ice cream (Bardsley, 1938; Nelson, 1944), meat and poultry (Haines, 1934; Sulzbacher, 1950; Kirsch *et al.* 1952; Eddy & Kitchell, 1959) and meat products (Jensen & Hess, 1941), and from other environments such as cold stored blood (Pittman, 1953) and faeces (Buttiaux, 1959). It is therefore obvious that such psychrotrophic Enterobacteriaceae are rather widespread.

The occurrence of such strains may seriously interfere with the hygienic evaluation of foods, as Eddy & Kitchell (1959) first noted. It is customary in the Netherlands to reject processed foods and meals which contain more than about 10 Enterobacteriaceae/g, because of the certainty that such levels do not arise from infection alone, but as a result of contamination followed by the potentially dangerous growth of bacteria during storage of the food (Mossel, 1959). This principle would not be valid, however, if the Enterobacteriaceae in question were psychrotrophic types, because these may develop quite rapidly even at temperatures below 5°, and these certainly cannot be called unsuitable for food storage. It was therefore imperative to look for a quick, though perhaps tentative, test which would provide a means of differentiating between normal and psychrotrophic Enterobacteriaceae. A direct test for capacity to grow at 2–5° cannot be used for this purpose, because its completion might require about two weeks. Therefore, an attempt was made to use their inability to develop at an elevated temperature (Eddy & Kitchell, 1959). To eliminate adventitious influences as much as possible, the criterion preferred was growth on a nonselective agar medium rather than gas formation in a selective liquid medium.

METHODS

Strains of bacteria. In this investigation 65 strains of Enterobacteriaceae were used. Twenty-six of these were authentic strains of the genera *Escherichia*, *Aerobacter*, *Citrobacter*, *Serratia*, *Salmonella*, *Shigella*, *Hafnia* and *Proteus*, which did not develop within 60 days at 6°, while the other 39 could do this and can therefore be considered psychrotrophic strains. Of the latter, 26 were isolated from ice cream and raw meat-snacks ('snack tartar') which had been stored for about 2 weeks at 2–3° by plating on crystal violet-neutral red-bile-lactose-mannitol (CNBLM) agar (Mossel, 1957). The other psychrotrophic Enterobacteriaceae were provided by Mr. S. B. Thomas, N.A.A.S., Trawscoed, Aberystwyth and Dr. B. P. Eddy, Low Temperature Research Station, Cambridge. They had been isolated from refrigerated milk and meat respectively.

Medium, inoculation and incubation. Fresh 30° cultures of all the strains were streaked on TDYM agar, a nonselective medium found to be very suitable for general subculturing by Mossel & Krugers Dagneaux (1959), and replicate tubes were incubated at 39·5, 42, 43 and 44°. In all tests, the cultures were incubated in electronically controlled ultrathermostats in which the temperature was maintained within 0·1° of the intended value.

RESULTS AND DISCUSSION

The results showed that whereas quite a few of the normal Enterobacteriaceae were inhibited at 44°, 42° still permitted growth of some of the psychrotrophic types; so $43 \pm 0{\cdot}1°$ was therefore the temperature of choice. At this temperature 23 of the 26 nonpsychrotrophic strains tested grew well within 24 hr, but none of the psychrotrophic types showed any macroscopically visible development even after 48 hr. The three mesophiles which behaved abnormally also showed other discrepancies. But should some normal nonpsychrotrophic strains of Enterobacteriaceae not grow at $43 \pm 0{\cdot}1°$ this would not be too serious, because it would never lead to the unwarranted rejection of foods. Conversely, however, the abundant growth of psychrotrophic types at 43° would do so.

This investigation confirms in principle both the observations of Novel (1947), who demonstrated that a temperature of about 43° inhibited none of the eight coli-aerogenes bacteria tested, and those of Eddy & Kitchell (1959), who reported the inhibition of all the psychrotrophic Enterobacteriaceae studied at about 44°. Therefore it seems justifiable to use this test in the tentative identification of Enterobacteriaceae isolated from foods, in the following way. The classical confirmatory reactions—morphology, nitrate reduction and glucose fermentation (Mossel & Krugers Dagneaux, 1959)—are extended to include a growth test on TDYM agar slants at $43 \pm 0{\cdot}1°$, which should be positive within 24 hr.

From the work of Prescott & Geer (1936) and Angelotti *et al.* (1959) it seems not impossible that, at least in certain foods, considerable numbers of psychrotrophic salmonellas might occasionally be detected with the help of the 43° test. Clearly, in these cases the samples concerned should be rejected, as is usually done with *Salmonella* positive commodities. Also, according to these authors it seems quite possible to suppress the growth of these salmonellas by lowering the cold storage temperature from 10° to 5° or below.

The authors wish to thank Mr. S. B. Thomas and Dr. B. P. Eddy for providing psychrotrophic isolates and for helpful discussions.

The present address of H. Zwart is: H. J. Heinz Company (Holland) N.V., Elst, Gld., The Netherlands.

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THE USE AND MEANING OF THE TERM ‘PSYCHROPHILIC’

By B. P. EDDY
Low Temperature Research Station, Cambridge

SUMMARY: A case is presented for the introduction of the term ‘psychrotrophic’ for bacteria able to grow at 5° or less. The term ‘psychrophilic’ should be used only when a low optimum temperature is implied.

In bacteriological literature the adjectives thermophilic, mesophilic and psychrophilic, and also the corresponding nouns, are used in a loose and ill defined manner. This is particularly true of the term psychrophilic. Ingraham & Stokes (1959) in their review of ‘psychrophilic bacteria’ refer more than once to the definitions given in textbooks of bacteriology. Those cited in Table 1 may be taken as examples of the many available; it will be seen that agreement is poor.

Among the earliest writers to use the term psychrophilic (ψῡχρός, cold; φίλος, loving) was Schmidt-Nielsen (1902), who defined psychrophiles as bacteria which are not only able to survive but also to multiply at 0°. This definition was immediately challenged by Müller (1903) on the ground that many such bacteria cannot correctly be termed psychrophilic since they have optimum temperatures between 20 and 30°. Nevertheless, in spite of Müller’s admonition, the term psychrophilic has continued to be applied to bacteria capable of growing at low temperatures, irrespective of their optimum temperatures. This may be due partly to the fact that whereas such organisms are almost invariably isolated following lengthy incubation at low temperatures, the optimum temperatures are determined only subsequently, if at all; and partly because few organisms have been shown to have optimum temperatures below 20° (Berry & Magoon, 1934; Ingraham & Stokes, 1959). Ingraham & Stokes (1959) have returned to Schmidt-Nielsen’s (1902) definition although, because of knowledge gained in the interim, they have been led to recognize a wide range of optimum temperatures (Table 1). Such was the type of situation which caused Frazier (1958) rightly to state the semantic paradox that, judged by their optimum temperatures, most psychrophiles would be considered to be mesophilic. It seems to the present writer that there is a need for a word carrying the sole implication of the ability to multiply, preferably on solid media, at temperatures of about 5° and below, within given experimental time limits (e.g. x days at 5°, y days at 0°, z weeks at –5°, etc.), without any references being made to optimum temperature. This might permit a redefinition of the word psychrophilic in terms more generally acceptable.

Various terms for cold-growing bacteria have been proposed in the past, e.g. *glaciale Bakterien*, psychrotolerant, eurythermic and thermophobic, but none has gained acceptance. The writer considers that the word psychrotrophic (τράφω, to increase, thrive), suggested by Dr. D. A. A. Mossel, should be used for bacteria able to grow at +5° and below, whatever their optimum temperatures.

It is undesirable that bacteria should be classified by their reactions to two temperatures lying as far apart as the minima and optima usually do. Hence, the term psychrophilic should be defined according to optimum temperature, to which the maximum limit is usually fairly close, without reference being made to minimum temperatures. Since it is clearly an advantage to carry out as few temperature-growth determinations as possible, it is tentatively suggested that psychrophiles be defined as bacteria with a maximum growth temperature below 35°.

Table 1. *Cardinal temperatures of psychrophilic bacteria*

Reference	Cardinal temperatures (°C)		
	Minimum	Optimum	Maximum
Eyre (1902)	0	15	30
Buchanan & Fulmer (1930)	—	18–20	—
Clifton (1951)	0	10–20	30
Ingram (1951)	<0	15–25	—
Oginsky & Umbreit (1955)	0	12–18	25
Jacobs, Gerstein & Walter (1957)	0	<15	30
Ingraham & Stokes (1959)	*c.* −10	20–40	<45

As examples of the use of these terms and the distinction between them there may be cited (1) the 'cold-tolerant' coli-aerogenes bacteria described by Eddy & Kitchell (1959) which would be called psychrotrophic mesophiles, and (2) *Staphylococcus afermentans* (NCTC 2676) which will not grow at 35°, which does not grow at 5°, and which would be psychrophilic but not psychrotrophic. There will, as with any classification based on temperature, be organisms which just transgress the border line and whose description must be a matter for individual judgment.

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THE INCIDENCE OF PATHOGENIC SEROTYPES OF *ESCHERICHIA COLI* IN PASTEURIZED MILK AND WASHED BOTTLES

By J. G. MURRAY

Ministry of Agriculture for Northern Ireland and
The Queen's University of Belfast

SUMMARY: During the period April–December 1959 some 7,834 samples of pasteurized milk were examined. From the positive presumptive results in MacConkey's broth at 37°, 165 cultures were obtained which fermented lactose at 44°. From 1957 to 1959, 11,400 washed bottles were tested and 694 gave a positive result in MacConkey's broth at 30°; only 12 of these cultures were positive at 44°. None of the strains of *E. coli* was a pathogenic serotype.

The literature on the subject of the coli-aerogenes group in milk is most comprehensive. The question of heat resistant coli-aerogenes bacteria in pasteurized milk has been discussed by several workers including Barkworth (1941), Crossley (1946) and Hiscox (1946, pers. comm.). Procter (1943) and Morris (1943) found heat resistant members of the coli-aerogenes group in raw milk, but it was not established if this was genetic in character as was the case with the culture investigated by Hiscox (1946). Crossley (1946) found coli-aerogenes bacteria after laboratory pasteurization in only 2 of 978 samples of milk. He stated that such organisms are rare in raw milk, but are found more frequently in pasteurized milk. Heat resistant strains are usually only found at points beyond the holder and are mainly due to plant contamination. He showed that 50% of the strains obtained from swabbings of the plant were heat resistant, and many of these were undoubtedly *Escherichia coli*. He suggested that inadequate washing and sterilizing may have had a selective and cumulative effect, and caused a build-up of such strains on the plant surfaces.

That heat resistant strains of coli-aerogenes bacteria do occur has been shown by the workers quoted; but Murray (1955, unpublished data), who examined many thousands of samples of both raw and pasteurized milk in Northern Ireland for the presence of such strains, was unable to demonstrate their presence. It thus seems reasonable to assume that the presence of coli-aerogenes bacteria in efficiently pasteurized milk is almost invariably a result of post-pasteurization contamination.

The coli-aerogenes flora of raw milk has been discussed in detail by Malcolm (1933, 1939) and Bartram & Black (1937). The types found in raw and pasteurized milk and other dairy products are fully described by Crossley (1946). A discussion of the coli-aerogenes group is made difficult by the varying and confused nomenclature used, and also by the great variations in technique and media used by different workers. However, a study of the literature revealed that a proportion of the strains present in raw and pasteurized milk were *E. coli*.

Taylor (1959) has traced the history of *E. coli* from its isolation in 1886 by Escherich, and the gradual change of ideas from considering it a harmless saprophyte to its acceptance as a causative agent of diarrhoea. Evidence has been accumulated by many workers of the relationship between infantile diarrhoea and the most important serotypes of *E. coli*: 0111.B4, 055.B5 and 026.B6. Milk has been suspect as a source of infection. The contamination of cows' milk could, according to Papavassiliou (1957), happen directly through mastitis due to *E. coli*, or via animal carriers, or incidentally via human carriers. This worker examined 375 samples of retail pasteurized milk sold in the London area from January to August 1955 and found that 10% of the samples contained *E. coli* I or II; but no pathogenic serotypes were found. He stated that several workers in Germany tried to find the organism in raw and pasteurized milks, but their results indicated that milk-borne infection is very rare.

The work described below was done in an attempt to determine whether pathogenic serotypes of *E. coli* were present in pasteurized milk (mainly bottled) in Northern Ireland, and also to discover whether washed milk bottles from all processing plants might contain these organisms.

EXPERIMENTAL METHODS

Samples of pasteurized milk taken from the plant or during the course of distribution were examined during the period April–December, 1959. Most of the samples were in pint or one-third pint bottles (schools supply) but some were taken from churns of milk for hotels, hospitals, etc. In all 7,432 samples were examined by the presumptive coli-aerogenes and phosphatase tests. All the processing plants used the HTST method of pasteurization.

Each sample was thoroughly mixed and 1 ml added to each of three test tubes containing 5 ml of MacConkey's broth. The tubes were incubated at 37° and examined after 18 hr and 48 hr incubation for acid and gas production. As soon as a positive result was observed a loop transfer was made to each of two tubes, one containing peptone water and the other brilliant green bile broth, and these were incubated in an accurately controlled water bath at 44° for 24 hr. The inoculations from the positive MacConkey's broth were made at as early a stage as possible in order to avoid overgrowth by any particular strain. After incubation at 44° for 24 hr the peptone cultures corresponding to positive brilliant green bile broth cultures were tested for indole (Goré modification). Dried MacConkey's neutral red agar plates were streaked from the peptone water cultures of those strains which gave positive reactions in the brilliant green broth and indole tests, and after 18–24 hr incubation at 37° the confluent growth on the plates was tested against *E. coli* polyvalent O serum using the slide agglutination technique. When a positive result was obtained, typical individual colonies were tested against the polyvalent serum. Colonies which gave a positive reaction were then tested against the following sera: types 026, 055, 0111, 0119, 0127 and 0128. When agglutination with a specific serum was obtained, the colony was transferred into peptone water, and after 6 hr at 37° the culture was inoculated into the appropriate media for lactose and inositol fermentation, the production of indole, the hydrolysis of urea and growth on citrate. Cultures which gave a positive indole reaction and fermented lactose were streaked on a Yeastrel-milk agar slope for further serological tests.

Washed bottles were tested for sterility by the technique recommended by the Ministry of Agriculture and Fisheries (1947). The inoculated MacConkey's broth was incubated at 30° and examined at regular intervals up to 72 hr. As soon as a positive result was observed a loopful was transferred to a tube of MacConkey's broth and incubated at 44°. The procedure adopted in the case of positive results was the same as that already described for pasteurized milk.

RESULTS

Pasteurized milk. The results of the examination of the 7,432 samples of pasteurized milk are given in Table 1, which shows the positive results at 37° and 44° for each successive month. Evidently there was a seasonal trend in the incidence of 37° and 44° positive cultures, the numbers of these being highest during the summer period.

Table 1. *The incidence of coli-aerogenes bacteria in pasteurized milk during nine successive months in* 1959

Month	No. of samples examined	Samples positive* at			
		37°		44°	
		No.	%	No.	%
April	890	115	12·9	5	0·6
May	922	139	15·1	0	0·0
June	891	181	20·3	12	1·3
July	720	187	26·0	36	5·0
August	571	159	27·8	23	4·0
September	947	230	24·3	18	1·9
October	834	151	18·1	52	6·2
November	933	129	13·8	9	1·0
December	724	103	14·2	10	1·4
Totals and percentages	7,432	1,394	18·76	165	2·22

* In one or more tubes.

Of the 165 strains of *E. coli* obtained none was a pathogenic serotype.

Washed bottles. The washed bottles examined during the period September 1957 to December 1959 totalled 11,400. Of these, 694 gave a positive presumptive result in MacConkey's broth at 30°, but only 12 (1·73%) gave a positive reading at 44° and none of these was a pathogenic serotype.

DISCUSSION

The results obtained in the present study confirm the findings of previous workers that pasteurized milk does not appear to be a source of pathogenic serotypes of *E. coli*. The large number of washed bottles examined also gave a negative result. However, the fact that 6·09% of the bottles gave a positive presumptive coli-aerogenes result at 30° cannot be regarded with satisfaction. A study of the results from individual processing plants showed that where management was good all the bottles gave a negative result in the presumptive test and it would appear to be a standard which can readily be reached.

With post-pasteurization contamination in milk assessed by the presumptive coli-aerogenes test, a small number of processing plants provided most of the positive results. When the mean shade temperature was high, the incidence of contamination was greatest. Nevertheless, the highest percentage of 44° positive types occurred in October, with July and August next in order.

With the few centres distributing pasteurized milk commonly containing coli-aerogenes bacteria, every effort should be made to lessen this contamination and so completely remove the already apparently negligible risk of contamination of pasteurized milk by pathogenic serotypes of *E. coli*.

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THE NORMAL INTESTINAL FLORA OF THE PIG. IV. THE EFFECT OF DIETARY SUPPLEMENTS OF PENICILLIN, CHLORTETRACYCLINE OR COPPER SULPHATE ON THE FAECAL FLORA

By R. FULLER, L. G. M. NEWLAND, C. A. E. BRIGGS,
R. BRAUDE and K. G. MITCHELL

National Institute for Research in Dairying, University of Reading

SUMMARY: Streptococci, lactobacilli and coli-aerogenes organisms were isolated from pigs receiving penicillin, chlortetracycline or copper sulphate as dietary supplements. Changes in their numbers, physiological type and sensitivity to the supplements fed are described. Penicillin and chlortetracycline rapidly induced a population resistant to the antibiotic fed, but no changes were detected in numbers or types of organisms isolated. Copper sulphate caused a reduction in the numbers of streptococci and a change of predominant type from non-lactose-fermenting to lactose-fermenting; there was also a change from a flora in which *L. acidophilus* predominated to one in which *L. brevis* and *L. cellobiosus* tended to become dominant.

The growth stimulation of pigs observed when antibiotics, as first demonstrated by Jukes, Stokstad, Taylor, Cunha, Edwards & Meadows (1950), or copper sulphate (Barber, Braude, Mitchell & Cassidy, 1955) are included in their diets, has subsequently been confirmed in experiments carried out in many different countries. In work on the mode of action of these supplements, many workers have observed changes in the intestinal microflora of pigs fed on diets containing antibiotics (Sieburth, Gutierrez, McGinnis, Stern & Schneider, 1951; Bridges, Dyer & Burkhart, 1952; Bridges, Dyer & Powers, 1953; Anderson, 1954). The results have been conflicting, due partly to lack of knowledge of the normal variations and composition of the intestinal microflora. More recently several workers (Briggs, Willingale, Braude & Mitchell, 1954; Willingale & Briggs, 1955; Fewins, Newland & Briggs, 1957; Raibaud, Caulet & Mocquot, 1957; Raibaud & Caulet, 1957) have shown that lactobacilli and streptococci are major components of the intestinal flora of the pig, with coli-aerogenes organisms present in smaller numbers.

In the present work changes in these three groups of organisms in the faeces of pigs, following supplementation of their diet with either antibiotics or copper sulphate, have been studied.

Materials and Methods

Pigs

A group of four Large White litter mates of similar weaning weights constituted an experimental unit. Shortly after weaning at 8 weeks, the pigs were put into individual pens and given the following diet: barley meal, 50%; fine millers' offal, 33%; decorticated ground nut meal, 15%; limestone, 1·5%; salt, 0·5%; vitamin A and D supplement ('Rovimix', Roche Products Ltd., containing 50,000 i.u. vitamin A and 12,500 i.u. vitamin D_3/g), 4·5 g/100 lb of diet.

After two weeks, supplementation of the diet was begun. One pig in each group received procaine penicillin, 10 g/ton; another received Aurofac 2A (Cyanamid of Great Britain, Ltd.: containing 3·6 g/lb of chlortetracycline), 3 lb/ton; and a third received $CuSO_4.5H_2O$, 0·1% (providing 250 p/m of copper).

One pig in each group was left on the unsupplemented diet, as the control. Four groups of four pigs were examined and one group of five, containing two pigs on the copper sulphate supplemented diet, making a total of 21 pigs. The pigs were continued on these diets until they reached an average weight of 208 lb (an experimental period of 13–19 weeks).

Samples of freshly voided faeces were collected at weekly intervals in clean waxed cartons. The number of samples collected before supplementation began varied from one to three. Samples from the caeca of all pigs were collected at slaughter and the close agreement between the numbers of streptococci, lactobacilli and coli-aerogenes organisms found in these and the faecal samples was confirmed (see Willingale & Briggs, 1955).

Bacteriological Methods

Media

Samples (2 g) of faeces were mixed with 18 ml of previously boiled quarter-strength Ringer's solution. Serial transfers (2 ml) were made in boiled quarter-strength Ringer's solution to give dilutions up to 10^{-9}. Five tubes of previously boiled Reinforced Clostridial Medium (RCM of Hirsch & Grinsted, 1954) were inoculated with 0·2 ml from each dilution and 5 tubes of MacConkey's broth were inoculated from each of the 10^{-4}–10^{-8} dilutions. After incubating for 3 days at 37°, Gram stains were made from the positive RCM tubes to determine the dilution tubes in which lactobacilli and streptococci were growing. Using probability tables (Hoskins, 1934), the number of organisms/g wet weight of faeces was calculated. Hoskins' tables were applied directly to the MacConkey's broth results.

Isolations were made on 5% horse blood agar from the top four positive tubes of RCM showing growth of streptococci, and on tomato dextrose agar (Briggs, 1953) from the top four positive tubes showing growth of lactobacilli. The highest positive tube of MacConkey's broth showing production of acid and gas was plated out on MacConkey's agar. Single colonies were picked off and freeze-dried. Frequently, some of the MacConkey's broth tubes showed only acid production; isolations were also made from these.

Typing of micro-organisms

All isolates growing as pairs or chains of catalase-negative, Gram-positive cocci were provisionally classified as streptococci, while all isolates growing as single, paired or chain-forming catalase-negative, Gram-positive rods were provisionally classified as lactobacilli.

Streptococci. Presumptive streptococci were characterized by: growth in dextrose-Lemco broth + 6·5% of sodium chloride; hydrolysis of starch; hydrolysis of arginine (Abd-el-Malek & Gibson, 1948); haemolysis of horse blood; sensitivity to 1:2500 potassium tellurite (after Sharpe & Shattock, 1952); gelatin liquefaction; effect on litmus milk; and carbohydrate fermentation (see Table 2). For the last mentioned test a final concentration of 0·2% carbohydrate in bromocresol purple peptone water was used and 0·02 ml of culture was inoculated into 5 ml. The tubes were incubated at 37° for 5 days and read after 1, 3 and 5 days.

More vigorous growth could be obtained by adding 1% of horse serum to the test media and this was adopted as routine practice without affecting the results of the various tests.

For serological tests HCl extracts of 18 hr cultures in 1% dextrose-Lemco broth were prepared after the method of Lancefield (1933) and concentrated by Shattock's (1949) technique. When these extracts gave negative results with sera of all groups, formamide extracts were prepared by the method of Fuller (1938).

The extracts were layered over group sera in tubes of 3 mm internal diameter and examined at periods up to 30 min. They were tested first against group D serum and, if this was negative, against sera for the other groups from A to O.

Lactobacilli. Presumptive lactobacilli were characterized by: production of gas from glucose (Gibson & Abd-el-Malek, 1945); production of ammonia from arginine (Briggs, 1953; modification of Niven, Smiley & Sherman, 1942); fermentation of carbohydrate (0·2%) in basal medium (see Wheater, 1955). The list of carbohydrates tested is given in Table 4. An inoculum of 0·05 ml was used and the tubes were incubated at 37° for 2 weeks. Readings were made at 3, 7 and 14 days. Growth tests were made at 15°, 45° and 48°. The presence or absence of growth at 15° was read at intervals up to 3 weeks, whereas the tests at 45° and 48° were read at periods up to 1 week.

Coli-aerogenes organisms. These were examined by 'IMViC' and Eijkman tests.

Sensitivity tests

All strains of streptococci and lactobacilli were tested against penicillin, chlortetracycline and copper sulphate. The coli-aerogenes organisms were tested against only chlortetracycline and copper sulphate.

The tests were done on 5% horse blood agar, tomato dextrose agar and nutrient agar for the streptococci, lactobacilli and coli-aerogenes organisms, respectively. The inoculum consisted of 0·04 ml (0·1 ml for lactobacilli) of a vigorously growing broth culture spread on the surface of the plate. 'Sentest' tablets (Evans Medical Supplies Ltd.) of penicillin, 0·5 and 2·5 i.u., and of Aureomycin (chlortetracycline), 10 and 100 μg, were used for the antibiotic sensitivity tests; a well plate method was used for copper sulphate sensitivity tests in which 0·02 ml of a copper sulphate solution was placed in wells cut in the medium with a sterile no. 4 cork borer. For streptococci and coli-aerogenes organisms the strength of the solution was 5% and 10%, while for lactobacilli it was 1% and 5%. These concentrations gave zones of inhibition comparable in size to those produced by the antibiotic tablets. The plates were incubated for 48 hr at 37° and organisms were recorded as resistant if confluent growth occurred up to the tablet or well.

RESULTS

Growth rate and efficiency of food utilization of pigs

The individual results for both liveweight gain and efficiency of food utilization for each pig are shown in Table 1. While some of the pigs showed the expected growth response to antibiotic or copper supplementation as demonstrated in the studies referred to earlier, others failed to exhibit any response.

Table 1. *Liveweight gain and efficiency of food utilization of individual pigs during the period from shortly after weaning at 8 weeks of age to bacon weight*

Pig nos.	Supplement (/ton)			
	None	3 lb Aurofac 2A†	10 g procaine penicillin	2·24 lb $CuSO_4.5H_2O$
1, 9, 5, 18	1·22* **3·26***	1·44 **3·00**	1·48 **3·01**	1·52 **3·04**
22, 17, 21, 20	1·34 **3·45**	1·48 **3·27**	1·40 **3·21**	1·39 **3·27**
4, 2, 3, 6	1·07 **3·69**	1·44 **3·01**	1·05 **3·39**	1·09 **3·68**
40, 46, 43, 39	1·44 **3·11**	1·44 **3·07**	1·33 **3·22**	—‡
24, 25, 28, 27	1·37 **3·31**	1·38 **3·34**	1·44 **3·35**	1·39 **3·26**
26				1·10§ **3·48§**
Means	1·29 **3·36**	1·44 **3·14**	1·34 **3·24**	1·36 **3·28**

* Each first line of figures shows live weight gain; bold figures show efficiency of food utilization (lb meal/lb. live weight gain).

† Containing 3·6 g/lb of chlortetracycline.

‡ This pig failed to grow normally and was taken off experiment before reaching bacon weight.

§ Additional pig on copper supplemented diet in the last group not included in mean results.

Bacteriological results

Quantitative changes. The five unsupplemented pigs examined served to establish a base line against which any changes coincident with feeding supplements could be evaluated. Streptococci, lactobacilli and coli-aerogenes organisms were confirmed as major constituents of the flora, being found, respectively, within the ranges 10^5–10^9, 10^5–10^9 and 10^4–10^7/g wet weight of faeces. Fig. 1 shows the results obtained with pig no. 1, which were representative of those from all the control pigs.

The results with chlortetracycline did not differ markedly from these. In three of the five pigs fed penicillin there was a transient reduction in the numbers of streptococci, while the numbers of lactobacilli and coli-aerogenes organisms were not significantly different from those in the controls (see Fig. 2).

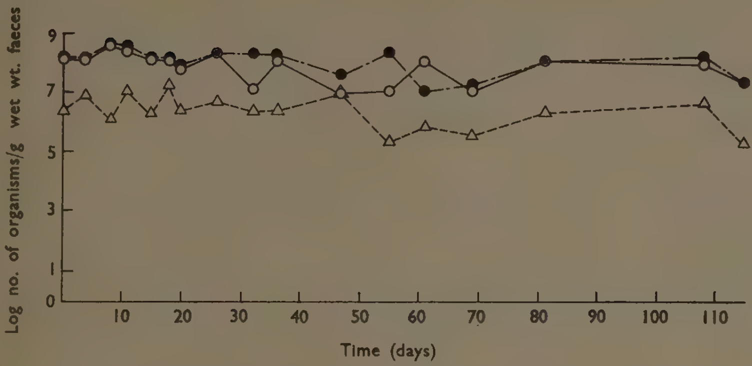

Fig. 1. Numbers of lactobacilli, streptococci and coli-aerogenes organisms in the faeces of a control pig (no. 1) during the experimental period.

Full circles, lactobacilli; open circles, streptococci; open triangles, coli-aerogenes organisms.

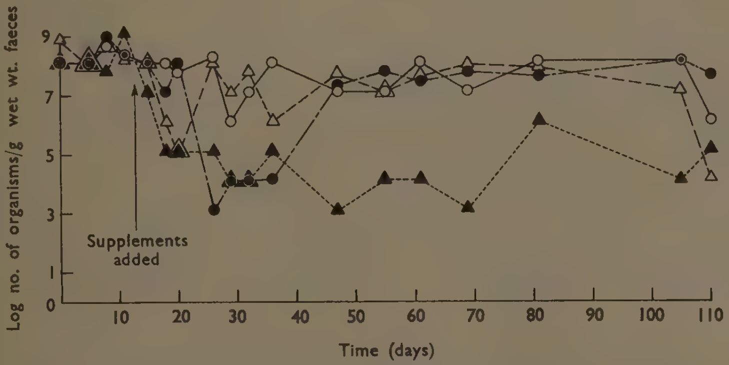

Fig. 2. Numbers of streptococci in the faeces of a group of pigs (control and supplemented) during the experimental period.

Open circles, control (pig no. 1); open triangles, chlortetracycline (pig no. 9); full circles, penicillin (pig no. 5); full triangles, $CuSO_4$ (pig no. 18)

In pigs fed copper sulphate the numbers of lactobacilli and coli-aerogenes organisms remained unaffected, but there was a marked fall in the numbers of streptococci. This reduction usually took place within a week of supplementation and lasted until the animals were slaughtered. The extent of the reduction varied, but the count of streptococci after supplementation of the diet was always lower than in the control and at times the reduction was as much as from 10^9 to 10^3 organisms/g wet weight of faeces.

Qualitative changes. All the streptococci isolated belonged to serological group D and, on the basis of the results of the biochemical tests, were classified into the species shown in Table 2.

Table 2. *Physiological characteristics of streptococci isolated from pig faeces*

	Characteristics* of strains							
	S1	S2	S3	S4	S5	S6	S7	S8
Number of isolates (total from all pigs 252)	160	54	14	7	8	5	2	2
Fermentation of:								
Arabinose	— (159)	— (52)	—	— (5)	—	+	—	+
Glycerol	—	— (51)	+	— (4)	—	+ (3)	—	+
Lactose	—	+	+	+	+	+	+	+
Mannitol	+ (112)	+ (41)	+	+ (5)	—	+	—	+
Raffinose	+ (130)	+ (45)	— (11)	— (4)	— (6)	— (3)	+	+
Action on litmus milk	—	A (35) AC (9) ARC (5) ACR (5)	RACP (5) ACRP (5) ARCP (2) RCP (1) ACP (1)	AR (1) RC (1) ARC (2) RAC (3)	ACR (2) RAC (4) ARC (2)	RC (1) ARC (2) RAC (2)	AC (1) ACR (1)	ARC (1) RAC (1)
Gelatin liquefaction	—	—		—	—	—	—	—
Growth in presence of 6·5% NaCl	—	— (51)	+	+	+	+	—	+
Hydrolysis of starch	—	—	—	—	—	—	+	—
NH_3 from arginine	—	—	+	+	+	+	—	—
β-haemolysis (horse blood)	—	—	—	—	—	—	—	—
Growth on 1/2,500 pot. tellurite agar	—	—	+	+	—	—	—	+
Classification	*Str. equinus*?	Unclassified	*Str. faecalis* var. *liquefaciens*	*Str. faecalis*	*Str. durans*	*Str. faecium*	*Str. bovis*	Unclassified

* A, acid; R, reduction; C, clot; P, peptonization. Figures in brackets show the number of strains giving the reaction indicated.

Table 3. *Lactose fermenting strains of streptococci in the faeces of control and supplemented pigs*

Pig no.	Dietary supplement	Lactose fermenters/ total isolates	% lactose fermenters
1	None (control)	2/9	22·2
9	Chlortetracycline	1/9	11·1
5	Penicillin	2/11	18·2
18	$CuSO_4$	6/8	75·0
22	None (control)	1/12	8·3
17	Chlortetracycline	1/13	7·7
21	Penicillin	0/14	0·0
20	$CuSO_4$	6/9	66·7
4	None (control)	7/13	53·8
2	Chlortetracycline	2/7	28·6
3	Penicillin	4/11	36·4
6	$CuSO_4$	8/8	100·0
40	None (control)	1/5	20·0
46	Chlortetracycline	5/10	50·0
43	Penicillin	4/8	50·0
39	$CuSO_4$	4/4	100·0
24	None (control)	2/12	16·7
25	Chlortetracycline	7/12	58·3
28	Penicillin	1/9	11·1
27	$CuSO_4$	9/9	100·0
26	$CuSO_4$	No strains isolated	

Most of the streptococci isolated from the control pigs were of the non-lactose-fermenting type S1, whose classification as *Str. equinus* is discussed later, and this was considered to be the 'normal' flora. On this basis penicillin and chlortetracycline at the levels fed had no effect on the streptococcal flora, but lactose-fermenting types were isolated more frequently from pigs receiving copper sulphate in their diet (see Table 3).

Table 4 shows the physiological characteristics of the lactobacilli isolated. The 168 strains were classified into the eight species shown. *Lactobacillus acidophilus* was the predominant lactobacillus in the faeces of the control pigs. The only marked and consistent change in the supplemented pigs was found in those receiving copper sulphate. In these there was a reduction in the number of *L. acidophilus* strains isolated, with a corresponding increase in the number of other types, especially *L. brevis* and *L. cellobiosus* (see Table 5).

Table 4. *Physiological characteristics of lactobacilli from pig faeces*

	Characteristics* of strains							
	L1	L2	L3	L4	L5	L6	L7	L8
Number of isolates (total from all pigs 168)	31	24	20	79	10	2	1	1
Gas from glucose	+	+	+	—	—	—	—	—
NH_3 from arginine	+	+	+	—	—	—	—	—
Growth at 15°	—	—	— (19)	—	—	+ (1)	—	—
Growth at 45°	+	+	+	+	+	+	+	+
Growth at 48°	—	—	— (16)	— (76)	— (9)	—	+	+
Fermentation of:								
Amygdalin	—	—	+	+	+	+	+	+
Cellobiose	—	—	+	+	+	+	+	—
Glycerol	—	— (23)	— (19)	—	—	+	—	—
Inositol	—	—	—	—	—	—	—	—
Lactose	+	+	+	+	+ (7)	+	+	+
Mannitol	—	—	— (14)	—	+	+	—	—
Melezitose	—	—	—	—	—	+ (1)	—	—
Melibiose	+	+	+	+ (68)	+ (9)	+	—	+
Raffinose	+ (26)	—	+ (15)	+ (65)	+ (8)	+	—	+
Salicin	— (30)	—	+	+ (76)	+ (9)	+	—	—
Sorbose	—	—	—	— (78)	—	—	—	—
Sucrose	+ (17)	—	+	+	+	+	+	—
Trehalose	—	—	— (19)	— (74)	—	+ (1)	—	—
Classification	*L. fermenti*	*L. brevis*	*L. cellobiosus*	*L. acidophilus*	*L. salivarius*	*L. plantarum*	Unclassified thermobacteria	

* Figures in brackets show the number of strains giving the reaction indicated.

The coli-aerogenes organisms isolated from tubes of MacConkey's broth showing acid and gas were *Escherichia coli* faecal type I. Isolates from tubes showing acid only were *E. coli* Irregular type I. There was no evidence from IMViC and Eijkman tests that supplementation of the diet had altered the coli-aerogenes population.

Development of resistant populations. At the lower concentrations of antibiotics cross resistance was common, but it can be seen in Table 6 that in general at the high concentration there was development of resistance to the antibiotic fed. In some instances there was an increase in the number of antibiotic resistant streptococci isolated from pigs fed copper sulphate. This was probably fortuitous, arising by the selection of the more resistant lactose-fermenting strains at the expense of the

Table 5. *Percentage distribution of types of lactobacilli in the faeces of control and supplemented pigs*

Pig no.	Dietary supplement	Types* of lactobacilli as % of total isolates							
		L1	L2	L3	L4	L5	L6	L7	L8
1	None (control)	16·7			83·3				
9	Chlortetracycline	12·5			75·0		12·5		
5	Penicillin	60·0			40·0				
18	$CuSO_4$	16·7	33·3	16·7	33·3				
22	None (control)				100·0				
17	Chlortetracycline			16·7	49·9	16·7			16·7
21	Penicillin				75·0	25·0			
20	$CuSO_4$	14·3	28·5	14·3	42·9				
4	None (control)	10·0		20·0	50·0	20·0			
2	Chlortetracycline	50·0			40·0			10·0	
3	Penicillin			10·0	70·0	20·0			
6	$CuSO_4$	66·7		33·3					
40	None (control)	28·5			57·2		14·3		
46	Chlortetracycline		33·3		66·7				
43	Penicillin		33·3		66·7				
39	$CuSO_4$	33·3	33·3	22·2	11·1				
24	None (control)	10·0	10·0	10·0	50·0	20·0			
25	Chlortetracycline	10·0		10·0	60·0	20·0			
28	Penicillin	10·0	10·0	40·0	40·0				
27	$CuSO_4$	10·0	50·0		20·0	20·0			
26	$CuSO_4$	8·3	50·0	16·7	8·3	16·7			

* See Table 4.

Table 6. *Sensitivity to penicillin and chlortetracycline (CTC) of strains of streptococci, lactobacilli and* E. coli *isolated from control and supplemented pigs*

Pig no.	Dietary supplement	Percentage* of resistant strains of									
		Streptococci, in presence of				Lactobacilli, in presence of				*E. coli*, in presence of CTC (μg)	
		Penicillin (i.u.)		CTC (μg)		Penicillin (i.u.)		CTC (μg)			
		0·5	2·5	10·0	100·0	0·5	2·5	10·0	100·0	10·0	100·0
1	None (control)	9·1	9·1	0·0	0·0	40·0	0·0	0·0	0·0	8·3	0·0
9	Chlortetracycline	33·3	0·0	44·4	0·0	50·0	25·0	50·0	25·0	87·5	50·0
5	Penicillin	80·0	80·0	0·0	0·0	100·0	60·0	20·0	0·0	0·0	0·0
18	$CuSO_4$	57·1	28·6	0·0	0·0	83·3	50·0	0·0	0·0	27·3	9·1
22	None (control)	0·0	0·0	18·2	0·0	100·0	0·0	0·0	0·0	12·5	0·0
17	Chlortetracycline	7·7	0·0	46·2	0·0	66·7	0·0	66·7	0·0	11·1	11·1
21	Penicillin	78·6	78·6	0·0	0·0	100·0	100·0	0·0	0·0	0·0	0·0
20	$CuSO_4$	54·5	36·4	27·3	0·0	85·7	0·0	28·6	0·0	0·0	0·0
4	None (control)	15·4	0·0	15·4	0·0	40·0	30·0	10·0	0·0	23·1	0·0
2	Chlortetracycline	0·0	0·0	50·0	0·0	75·0	0·0	50·0	0·0	55·5	44·4
3	Penicillin	90·9	63·6	18·2	9·1	100·0	80·0	0·0	0·0	27·3	0·0
6	$CuSO_4$	71·4	14·3	42·9	14·3	66·7	8·3	25·0	8·3	16·7	16·7
40	None (control)	16·7	16·7	0·0	0·0	42·9	14·3	71·4	14·3	0·0	0·0
46	Chlortetracycline	71·4	14·3	57·1	0·0	16·7	0·0	83·3	16·7	77·7	83·3
43	Penicillin	77·7	44·4	33·3	0·0	100·0	60·0	20·0	0·0	0·0	0·0
39	$CuSO_4$	0·0	0·0	0·0	0·0	25·0	12·5	25·0	12·5	25·0	0·0
24	None (control)	7·1	7·1	42·9	0·0	10·0	0·0	50·0	0·0	15·4	0·0
25	Chlortetracycline	7·7	7·7	46·2	0·0	11·1	0·0	66·7	0·0	92·3	38·5
28	Penicillin	100·0	12·5	12·5	0·0	100·0	83·3	33·3	8·3	21·4	0·0
27	$CuSO_4$	0·0	0·0	36·4	0·0	18·2	0·0	27·3	0·0	0·0	0·0
26	$CuSO_4$	No strains isolated				36·4	36·4	81·9	36·4	0·0	0·0

* Based on 4–14 isolates.

relatively sensitive non-lactose-fermenters. Two pigs fed copper sulphate also yielded antibiotic resistant lactobacilli but this also is related to changes at the species level rather than to an increase in resistance of particular strains. None of the organisms isolated showed resistance to copper sulphate at the concentration used and the results are therefore not included in Table 6.

DISCUSSION

It is recognized that not all the pigs in experiments of this nature respond to dietary supplements of antibiotics or copper. The reasons for this are not fully understood and a variety of factors may be involved. The failure of some of the pigs in the present studies to show a response is not, therefore, unusual, but it makes the interpretation of the bacteriological findings more difficult.

Previous studies in this laboratory, using pigs kept under the same conditions as the control animals in the present work, showed that the predominant streptococci were lactose-fermenters, and that the predominating lactobacilli were *L. acidophilus* and *L. fermenti* (Fewins, Newland & Briggs, 1957). The latter result has been confirmed but the predominant streptococcus in the present control pigs was found to be a non-lactose-fermenting type resembling *Str. equinus*. It is possible that this change was associated with the fact that the composition of the control diet was different in the present experiment from that used in earlier work. However, in those previous studies, when the diet was changed from one containing an animal protein supplement to one directly comparable to that used throughout in the present work, no such change was observed. Since all other factors of management and environment were identical in the two sets of experiments it may be that, even under given conditions, there is not necessarily a stable 'normal' streptococcal flora in the pig.

The difficulty of assigning a species name to group D streptococci is well known (see Shattock, 1955) and a similar difficulty has also been experienced with lactobacilli isolated from the intestine (see Fewins, Newland & Briggs, 1957). The two unclassified streptococci S2 and S8 are typical of the aberrant forms frequently isolated. S2 is a raffinose fermenting strain belonging to serological group D. However, the fact that it does not hydrolyze starch and ferments mannitol indicates that it is not *Str. bovis*. S8 grew in the presence of 1:2500 potassium tellurite, which is usually considered to be inhibitory to all group D streptococci except *Str. faecalis*. However, the other biochemical characteristics of S8 are inconsistent with its classification as this species.

The type S1 resembles closely those strains isolated from the intestine of the pig by Ribaud & Caulet (1957), which they classified as *Str. bovis*. The difficulty of distinguishing between *Str. equinus* and *Str. bovis* has been discussed by Seeley & Dain (1960). Their strains of *Str. equinus* hydrolyzed starch on plates whereas earlier workers (Hodge & Sherman, 1937) described *Str. equinus* as non-starch-hydrolyzing. This latter result has been confirmed with 3 strains from culture collections (American Type Culture Collection no. 1037, and National Collection of Dairy Organisms nos. 1090 and 1091). The inability to ferment lactose and hydrolyze starch would seem to preclude classification as *Str. bovis*. The one remaining factor against classification of the strains isolated from pig faeces as *Str. equinus* is their serological grouping.

Breed, Murray & Smith (1957) state that no group specific antigen has been demonstrated for *Str. equinus*, whereas the three strains referred to above all precipitated with group D serum when concentrated extracts (Shattock, 1949) were used; moreover, there was good agreement in the results of other tests between the three culture collection strains and those isolated from pigs.

The lack of effect of penicillin and chlortetracycline on the bacterial counts, although satisfactory levels of growth stimulation in a proportion of the pigs were produced, was surprising. However, the rapid development of resistance by the organisms to the antibiotics was, as expected, very evident and shows that these supplements had some effect on the flora. But, with either commensals or frank pathogens, this is not always the outcome, and Smith (1959) failed to find resistant strains of *Clostridium welchii* in pigs fed penicillin.

The changes in the flora of the pigs receiving copper sulphate cannot be correlated with the growth stimulating effect of this compound. The reduction in numbers of the streptococci and the change of type of the streptococci and lactobacilli occurred whether or not the pigs showed a response to the supplement.

In drawing conclusions the limitations of these studies must be borne in mind. The absence of any effect of dietary penicillin or chlortetracycline on these three components of the flora in no way suggests that the growth stimulating effect is not mediated through the gut flora. Nor does the absence of any correlation between the effects observed and the growth stimulation of pigs given dietary copper sulphate mean that the changes may not be involved in the mechanism of growth stimulation, since the failure of some of the pigs to exhibit any improved growth may have been due to other factors.

The requirements for 8 vitamins of the B-complex (aneurin, riboflavin, pantothenic acid, nicotinic acid, pyridoxine, folic acid, biotin and vitamin B_{12}) of some of the S1 strains of streptococci isolated before and towards the end of the period of supplementation were determined. No differences were found, but qualitative similarities in requirements do not preclude the possibility that dietary supplements could modify the quantitative requirements. The elucidation of this point must await the development of suitable techniques.

We wish to thank Mr. R. S. Barber for assistance with the experimental animals, Dr. J. E. Ford for the determination of vitamin requirements and Miss M. A. Shears for technical assistance. We also wish to thank the Directors and Staff of the Denny Group for facilities given at their Reading Factory.

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BACTERIAL MULTIPLICATION IN PACKED WILTSHIRE BACON

By M. INGRAM

Low Temperature Research Station, Cambridge

SUMMARY: Matured Wiltshire bacon normally carries 10^5–10^6 viable bacteria/g when sliced, predominantly salt tolerant micrococci and lactobacilli.

Kept at 15°, the numbers rose in 4 days to 10^7–10^8/g, the flora remaining generally similar in nature and with higher counts at 20° than at 37°. The situation then stabilized; there was a slow decline in organoleptic quality, but nothing unusual occurred in two weeks. Kept at 37°, similar numbers were reached after 1 day, and the nature of the flora changed rapidly, counts at 37° much exceeding those at 20°. Organoleptic deterioration was marked after 2 days and the bacon was unacceptable after 4 days. These changes occurred whether the bacon was packed in air or in 5% oxygen, the oxygen being all consumed in 4 days or less according to temperature. Apparently, if the bacon is kept at too high a temperature for its normal psychrophilic (20° optimum) flora, this is replaced by a mesophilic (37° optimum) flora. It appears that pathogenic staphylococci might be present in this mesophilic flora. A high proportion of isolates proved to be phosphatase positive, or 44° positive; and some had both these properties. Though all, so far, have been coagulase negative, there is no evident reason why pathogenic types should not occur.

Among the foods now marketed vacuum packed in film, bacon is one of the most popular. Because ordinary Wiltshire bacon is not normally a highly perishable commodity there is a tendency to assume that one can simply take any bacon, vacuum pack the slices in a bag, and all will be well: no special hazards will arise, and the bacon may (it is hoped) even keep longer than usual.

The better informed manufacturers are aware that such bacon is best kept in a cool place, and mark their packets accordingly. But experience with pasteurized canned hams, which bear similar instructions, shows that such recommendations are often disregarded (Ingram & Hobbs, 1954). Moreover, many manufacturers and wholesalers, who wish to adopt vacuum packing, are quite unaware of the considerations involved. Accordingly, a description is now presented of part of a series of investigations on the packing of bacon in this way: it deals particularly with the result of storing such bacon at too high a temperature, which might—it appears—encourage the multiplication of food poisoning staphylococci.

Materials and Methods

As part of the investigation was concerned with gaseous exchanges of the bacon, it was packed in cans instead of film bags, to facilitate the withdrawal of gas samples for analysis.

Normal mature Wiltshire bacon (back cut) was got direct from a factory and sliced in the laboratory. Six randomly chosen slices (150–200 g) were laid on a strip of cellophane, rolled endwise, and the roll was pushed into an A1 can. A volume of about 100 ml remained empty, which in different cans was filled with different atmospheres at normal pressure. One gas mixture, containing 95% N_2+5% O_2, aimed to reproduce an oxygen tension similar to that likely to be attained with a poor commercial vacuum.

The cans were then stored at 15° (representing normal temperature) or 37°. Individual cans (for reasons of economy) were taken at intervals, and samples of more than 25 g—mixed lean and fat from each slice—removed and homogenized aseptically. The rest was used for chemical and organoleptic analysis: but to give time to get bacteriological indications first, the samples were stored for 2–3 days at –3° before tasting.

Dilutions were made in quarter-strength Ringer's solution. The bacon was first homogenized in 4 volumes of diluent: then drops (0·025 ml) of appropriate dilutions were spread on quarters of each of duplicate plates. Average counts have been calculated per g (fresh weight) of bacon.

The culture media used were as follows. TDA: Hartley's tryptic digest of beef (Mackie & McCartney, 1946, p. 101) solidified with 2% agar, which could be modified in various ways. TDA plus NaCl: this was TDA with the addition of 3, 5 or 10% of salt, which makes the medium selective for micrococci. TDA plus crystal violet: 2 p/m of dye were added to TDA, to select Gram-negative rods (Holding, 1954). PP: phenolphthalein phosphate agar, which was TDA with the addition of 0·01% of phenolphthalein phosphate, was used to detect presumptively pathogenic staphylococci (Barber & Kuper, 1951). Tomato juice-thallous acetate(D): this was the medium of Sharpe (1955), which selects lactobacilli (and streptococci).

The temperatures at which these media were used are given later, with the counts. Plates incubated at 44° were counted after 1 day, those at 37° after 2 days; plates incubated at 20° were counted after 4 days, at which time staphylococci are not included in the count on the TDA-salt agars, though they will grow on longer incubation at that temperature (cf. Haines, 1934).

For the phosphatase reaction, the liberated phenolphthalein was detected by exposure of the plates to NH_3 gas. To permit further investigation of the organisms so counted, replicate plates not treated with NH_3 were prepared.

The coagulase reaction was determined by the tube method of Fisk (1940), using Wellcome citrated rabbit plasma diluted 1:5. As controls for each series, two coagulase positive staphylococci (NCTC 7447 and 8532) and two known negatives (NCTC 7292 and 7564) were run together with two uninoculated tubes. Final readings were made after 4 hr.

RESULTS

It is helpful, at the outset, to have some idea of the normal bacteriological condition of Wiltshire bacon, and Table 1 gives a few observations on bacon from the same factory, referring to the part of the side from which the experimental slices were taken. On emerging from the curing tank the bacon already carried a surface load of 10^4–10^5/cm^2, which rose rather more than tenfold during a week of maturation at 4° (there were far fewer bacteria within the side). The flora (cf. Ingram, 1934) consisted of salt tolerant micrococci, which appeared on TDA containing 3 and 10%

of salt, plus a roughly similar number of lactobacilli (which appeared on the tomato juice-thallous acetate medium) and a smaller proportion of Gram-negative rods (counted on the TDA-crystal violet medium). These are plainly cold tolerant (psychrophilic) bacteria, since they have multiplied quite rapidly below 5°. This surface load of psychrophiles is spread over the slices when the bacon is cut up.

Table 1. *The development of bacteria on the surface of two sides of bacon, during maturation at* 4°

Sample site	Time out of tank (days)	Side	Log$_{10}$ (colony count/cm^2) on medium* A	B	C	D	Mean log count
Long. dorsi	0	a	4·81	4·57	2·93	3·93	4·07
		b	4·57	4·49	1·60	4·03	3·90
	7	a	6·73	6·74	5·33	6·06	6·21
		b	5·16	5·15	5·03	5·79	5·28
Skin	0	a	5·10	4·66	2·81	4·15	4·18
		b	5·61	5·58	3·23	5·24	4·91
	7	a	6·88	6·76	4·11	6·16	5·88
		b	6·28	6·13	5·61	6·69	6·18

* Plating medium: TDA, tryptic digest of beef agar; A, TDA+3% NaCl; B, TDA+10% NaCl; C, TDA+crystal violet; D, tomato juice-thallous acetate. Plates incubated at 25°.

Table 2. *A comparison of colony counts on the contents of replicate cans of bacon, immediately after packing*

Medium*	Incub. temp. (°C)		Mean colony counts† for can no. 1	2	3
TDA	20		$7{\cdot}9\times10^4$	$3{\cdot}3\times10^4$	$2{\cdot}0\times10^6$
TDA	37		$2{\cdot}2\times10^4$	$4{\cdot}4\times10^3$	$4{\cdot}0\times10^5$
TDA+5% NaCl	20		—	$2{\cdot}5\times10^4$	$1{\cdot}9\times10^6$
TDA+5% NaCl	37		—	$7{\cdot}7\times10^3$	$1{\cdot}4\times10^6$
PP	37	Total	$3{\cdot}5\times10^3$	$3{\cdot}7\times10^3$	$2{\cdot}5\times10^5$
		Positive	$<10^2$	$<10^2$	$6{\cdot}0\times10^2$
PP	44	Total	$3{\cdot}0\times10^5$	$<10^2$	$1{\cdot}5\times10^3$
		Positive	$1{\cdot}0\times10^2$	$<10^2$	$2{\cdot}0\times10^2$

* TDA, tryptic digest of beef agar; PP, phenolphthalein phosphate agar.
† Means of two plates.

Turning now to the experimental canned bacon, the first question was how far data from individual cans might be regarded as representative. Three cans were accordingly examined immediately after packing, with the results shown in Table 2. Replicate plates agreed so well that it has been thought unnecessary to quote the individual figures and all the counts are averages for two plates. It is clear that there were large differences between the cans, both in the total numbers of various organisms present and in the proportions between them. Hence, in examining the data from the individual cans, one can regard as significant only broad trends, substantiated by observations from several cans either in succession or in parallel experiments. This must be borne in mind when considering the data which follow.

The viable counts for the bacon in the air-filled cans are shown in Fig. 1. Regarding first the cans stored at 15°, the numbers at 20° were at the outset about 10 times those at 37°, and this relationship was roughly maintained throughout the period of storage, though this eventually exceeded 4 weeks. When the cans were stored at 37°, however, a very different situation rapidly developed: after about 2 days, the counts at 37° increasingly preponderated over the counts at 20°. These broad conclusions are supported by the very similar results with the bacon packed in 5% oxygen instead of air (20% oxygen), shown in Fig. 2. Note first, in passing, that at the lower oxygen tension there was no great increase in the time which elapsed (about 4 days) before the maximum number of bacteria was reached, and a significant point, which was demonstrated by gas analyses, is that all the oxygen in these cans had already disappeared after 2 days storage at 37° or 4 days at 15°. These observations indicate that the storage life is not likely to be much improved save by a good vacuum, if at all; but detailed consideration of the effect of the atmosphere is out of place here. The important matter, in regard to the present theme, is that the contrast between bacon stored at 15° or at 37° was repeated: again, under warm conditions, a new mesophilic flora established itself in two days even with the lower initial oxygen content.

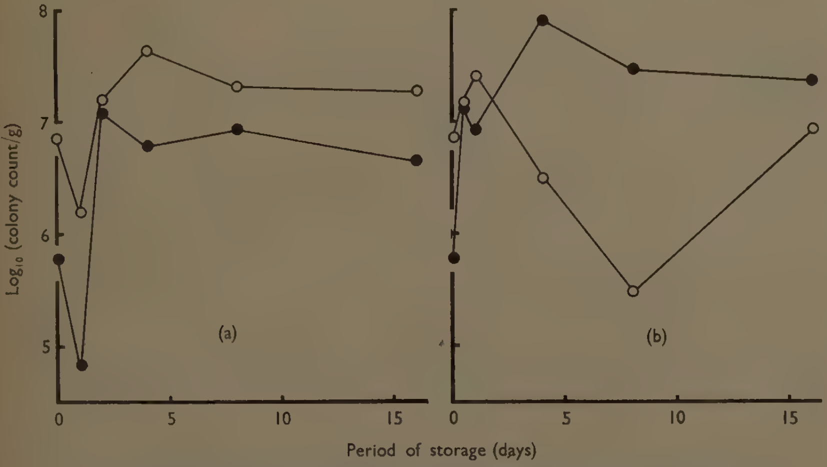

Fig. 1. The development of bacteria on sliced bacon packed in cans in air and stored at 15° (a) or 37° (b), as judged by colony counts on TDA+5% NaCl at 20° (open circles) or at 37° (full circles).

These observations plainly suggest that if bacon is stored at moderate temperatures it continues to carry its psychrophilic flora, whereas if it is stored at temperatures too high for that flora, above perhaps 30°, that flora is replaced in a day or two by a different, mesophilic, flora. This notion is confirmed by the quality changes in the bacon. The observations made by a taste panel of 10, of the odour uncooked and of the flavour after frying, are presented in Fig. 3. It is remarkable that when the bacon was kept at 15° its quality was not seriously affected for a fortnight, even though throughout that time it carried very large numbers of bacteria, as Fig. 1 has shown: this agrees with the idea that the bacteria were of a kind not radically different from those normally present during maturation. By contrast, when the bacon was stored at 37°, 'off' flavours and odours were obvious after 2 days and became pronounced by the time the mesophilic flora was strongly predominant.

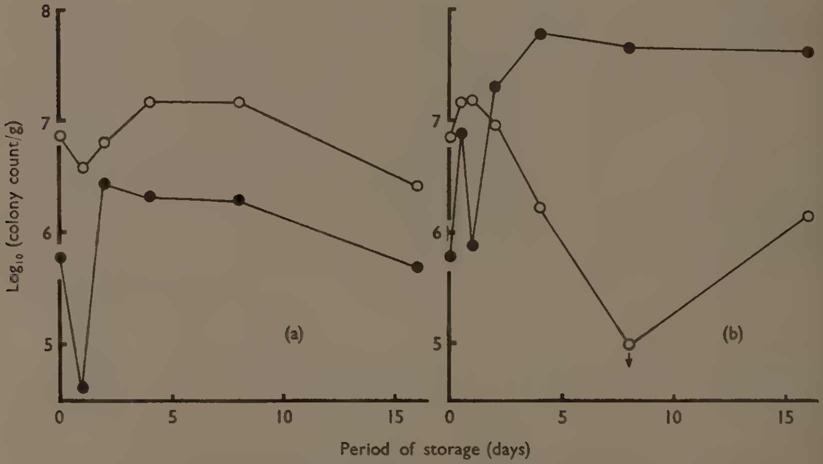

Fig. 2. The development of bacteria on sliced bacon packed in cans in 95% N_2+5% O_2 and stored at 15° (a) or 37° (b), as judged by colony counts on TDA+5% NaCl at 20% (open circles) or at 37° (full circles).

The indications concerning the nature of these mesophilic micro-organisms, which replace the normal flora if the bacon is kept too warm for a day or so, are disturbing: the organisms might apparently be staphylococci. To select staphylococci on artificial media sodium chloride at a concentration near 7·5% (Chapman, 1945: Maitland & Martyn, 1948), virtually equivalent to that found in normal bacon (when computed on the aqueous phase—cf. Oxhøj, 1943), is used. The pathogenic staphylococci are facultatively anaerobic, in contrast to the allegedly aerobic saprophytic micrococci (Breed, Murray & Smith, 1957); hence vacuum packing should encourage the former. *Staph. aureus* reduces nitrate, is proteolytic, and has a temperature optimum for growth near 37°. A warm vacuum pack of bacon looks indeed very like an unintentional selective medium for pathogenic staphylococci.

The investigation of this question is complicated by the difficulties surrounding the distinction of the genera *Staphylococcus* and *Micrococcus*. Setting aside the problems of nomenclature, what means are available for distinguishing the pathogenic from the nonpathogenic organisms? The problem is especially difficult with bacon, salt being useless here to select the pathogenic staphylococci, because all organisms growing on bacon must be adapted to a salty medium. But the pathogenic staphylococci will grow with no more than 0·5% of salt, while some bacon micrococci will not, and the former also grow at 44°—a temperature known to be inhibitory to the normal flora of bacon. This suggested a combination of the phosphatase test on a low salt medium and ability to grow at 44° as a hopeful means of detecting presumptively pathogenic staphylococci among the bacon flora, with confirmation by the coagulase test in the usual way. Observations on that basis are shown in Fig. 4.

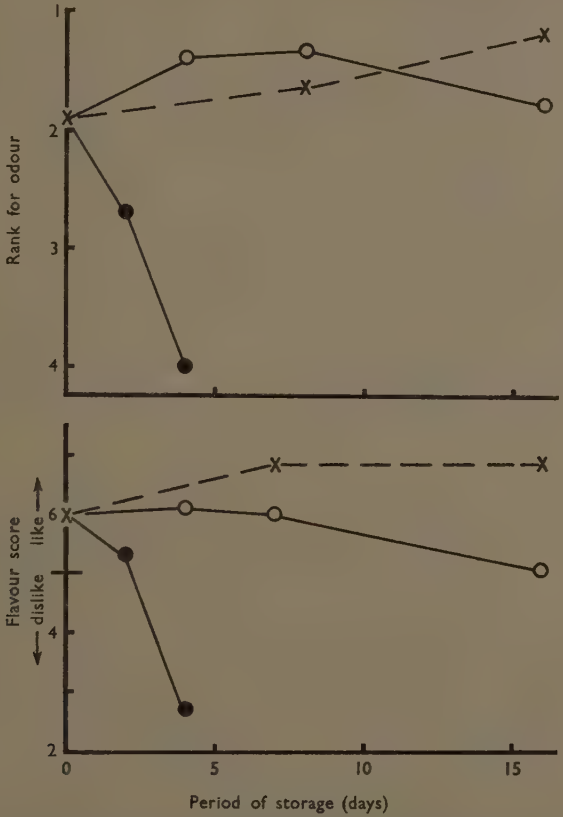

Fig. 3. The changes in odour (raw) and flavour (after frying, max. value 10) of sliced bacon packed in cans in 95% N_2+5% O_2 and stored at 15° (open circles) or 20° (full circles), assessed by a taste panel of ten members following the procedures of Coleby, Ingram & Shepherd (1959). Crosses, bacon stored at −3°, for comparison.

If all the organisms present were pathogenic staphylococci, the counts at 37° and at 44° should be the same, and all the colonies should be phosphatase positive. This was clearly not the case in the bacon stored at 15°; there was roughly a thousandfold preponderance of organisms neither 44° nor phosphatase positive. But in the bacon stored at 37° the 37° and 44° counts did become similar after about 2 days and many of these organisms were also phosphatase positive. Later, the proportions of 44° and/or phosphatase positive isolates fluctuated considerably, and it is evident that little quantitative significance can be attached to any individual observation. The important point is nevertheless plain: the flora of the bacon stored at 37° included large numbers of organisms either phosphatase positive or 44° positive or both. In this general respect the situation was again essentially similar with bacon packed in 5% instead of 20% oxygen (Fig. 5).

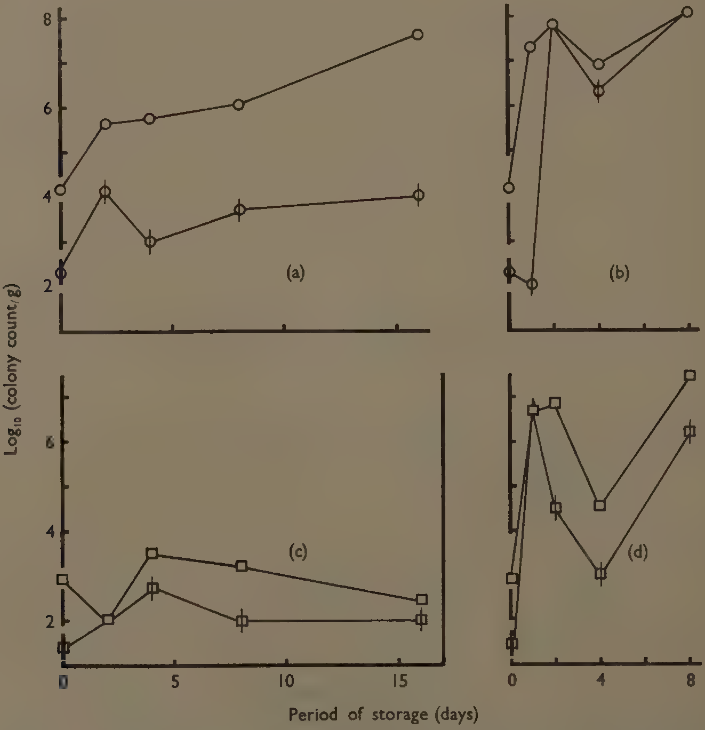

Fig. 4. The development, on sliced bacon packed in cans in air and stored at 15° (a, c) or 37° (b, d), of bacteria able to grow at 37° (circles) and 44° (squares) and able to form phosphatase at those temperatures (crossed symbols), as judged by colony counts on PP agar plus 0·5% NaCl.

To investigate the nature of these organisms further, colonies were picked from the following contrasted situations, using replicate plates which had been retained to permit isolation of colonies not tested for phosphatase with ammonia, namely where all the colonies in the phosphatase plate had been positive (Fig. 4, bacon kept at 37° in air for 2 days, count at 37°), and where they were predominantly phosphatase negative (Fig. 5, bacon kept at 37° in 5% O_2 for 1 day, count at 37°). In all cases the colonies were of catalase positive cocci resembling staphylococci in morphology.

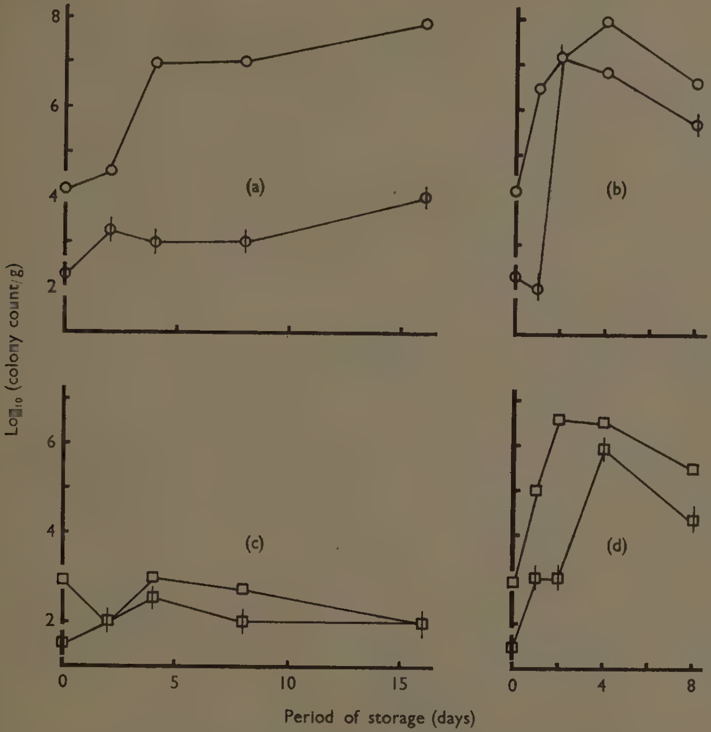

Fig. 5. The development, on sliced bacon packed in cans in 95% N_2 plus 5% O_2 and stored at 15° (a, c) or 37° (b, d), of bacteria able to grow at 37° (circles) and 44° (squares) and able to form phosphatase at those temperatures (crossed symbols), as judged by colony counts on PP agar plus 0·5% NaCl.

From the former situation 14 colonies were selected, of which 13 belonged to group II of Shaw, Stitt & Cowan (1951) and one to their group III (this was phosphatase positive). From the latter situation 22 colonies were picked, of which 5 proved to belong to group II and 17 to group III (all phosphatase negative). None of these organisms was coagulase positive, however; and as a further and incidental indication, 10 people had tasted the bacon stored at 37° without ill effect. It appears then that, with this sample of laboratory sliced bacon, the warmth induced flora did not consist of pathogenic staphylococci, though many of the organisms resembled them in respects other than coagulase reaction.

Discussion

From the above observations it appears that Wiltshire bacon which has been normally cured and matured is not a particularly suitable material for vacuum packing if it is packed without special precaution. The slices enter the pack with a high load of bacteria, which multiply to a maximum level in about 4 days at room temperature. The rate of this multiplication is not seriously limited by substantial reduction of the oxygen tension, which is scarcely surprising because most of the micrococci on bacon can get oxygen from nitrate instead of air (Ingram, 1952), and the lactobacilli do not need it.

It seems, moreover, that casual storage of such bacon under warm conditions might conceivably lead to food poisoning, if the bacon were infected with toxin forming staphylococci. The chances of such infection seem high, considering the many hand manipulations involved in the normal curing of bacon, where the chief protective agent is salt which staphylococci resist. The likelihood of human staphylococcal contamination would obviously be much increased under casual conditions of slicing and packing.

With this background, and knowing that sliced bacon must sometimes be stored domestically in undesirably warm conditions, it seems remarkable that bacon is practically never reported as a cause of food poisoning, because many people eat it only lightly cooked. Possibly there is some constituent of bacon, e.g. salt or nitrite, which can prevent formation of the staphylococcal toxin. (If so, it might interfere with the production of coagulase in artificial media, just as too much salt was shown to interfere with phosphatase by Barber, Brooksbank & Kuper, 1951. But it should be noted that the colonies used for the phosphatase and coagulase tests here were taken from PP agar which contained only 0·5% NaCl.

Investigations to reveal the incidence of pathogenic staphylococci on bacon are hindered by especial difficulties in distinguishing them from the predominant micrococci. As already mentioned, salt is here without selective value. As a further difficulty, Barber & Kuper (1951) advocated the use of phenolphthalein phosphate on the basis (Barber, Brooksbank & Kuper, 1951) that there is a close correlation between the phosphatase and coagulase reactions (and by inference pathogenicity); but on the bacon stored at 37° there were large numbers of organisms which were phosphatase positive but coagulase negative, and further investigation is evidently necessary. These bacteria appear to be similar to pathogenic staphylococci, save in their lack of coagulase (the character distinguishing group II from the pathogenic group I in Shaw, Stitt & Cowan's classification).

What view is to be taken of the development of large numbers of such bacteria when the bacon is kept warm? Though they are harmless in themselves, apparently, they can for the present be regarded as a 'presumptive staphylococcal count', indicating the existence of undesirable circumstances in which a dangerous situation could arise. But bacon is seldom exposed to temperatures as high as 37°, and there is a further insurance in the fact that when the undesirable organisms are numerous the bacon evidently becomes less attractive to the consumer. At lower temperatures, though staphylococci should be able to grow slowly, they are presumably crowded out by the better adapted psychrophiles. If that were the only protective factor, however, bacon specially produced with a low psychrophilic count to endow it with a long storage life but then exposed to staphylococcal contamination during packing, might develop a dangerous flora even if kept at moderate temperatures. The suspicions here raised can only be allayed by showing that, for unforeseen reasons, pathogenic staphylococci are unable to grow in suitable vacuum packed bacon, or are unable to produce toxin in those circumstances.

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CEPHALOTRICHOUS, FERMENTATIVE GRAM-NEGATIVE BACTERIA: THE GENUS *AEROMONAS*

By B. P. EDDY

Low Temperature Research Station, Cambridge

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1. Summary

The literature on the history of the genus *Aeromonas* has been reviewed and 46 strains have been examined. They showed marked similarities with respect to their ability to ferment carbohydrates, their lytic powers and their oxidase reaction. It is suggested that the genus *Aeromonas* Kluyver & van Niel should be re-defined and that it should comprise three species: *Aer. liquefaciens* (Beijerinck, 1900) Kluyver & van Niel, 1936; *Aer. salmonicida* (Lehmann & Neumann, 1896) Griffin, 1954; and *Aer. formicans* (Crawford, 1954) Pivnick & Sabina, 1957.

Four strains of *Kluyvera* have also been examined. They were found to be peritrichous.

2. INTRODUCTION

Gram-negative bacteria which resemble coli-aerogenes organisms in their fermentative ability, but which have polar flagella, have only recently become of interest to food and medical bacteriologists, though they have for many years been the concern of those interested in the diseases of fish and amphibia. In the last decade, however, strains have been isolated from diseased humans (Hill, Caselitz & Moody, 1954; Kjems, 1955) and from chilled meat (Eddy & Kitchell, 1959), and there is a report that *Aeromonas* plays an important role in the manufacture of raw sausage (Buttiaux, 1959).

This paper provides a review of the literature appertaining to this genus and the possibly related genus *Kluyvera*, and a description of the results of a comparative study of presumptive strains. From these two standpoints an attempt has been made to assess the status and relationships of this group of organisms.

3. A SURVEY OF THE LITERATURE ON THE GENERA *AEROMONAS* AND *KLUYVERA* AND RELATED ORGANISMS

The selection of the features discussed may at first glance seem arbitrary but this is largely due to the fact that many of the earlier descriptions of organisms now considered to belong to this group were very meagre. The topics chosen are those which were thought most likely to contribute to an assessment of the homogeneity, systematic relationships and importance of the group, namely an outline of the histories of the two genera and consideration of their flagellation, biochemistry, temperature relations, pathogenicity and serology.

(a) *The genus* Aeromonas

The genus *Aeromonas* is included in the 7th edition of Bergey's *Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957) and the definition of the genus is given as follows:—

"Short (rarely more than 3 microns), rod-shaped cells. Motile by means of polar flagella, usually monotrichous; occasionally non-motile. Gram-negative. Heterotrophic, oxidizing various organic compounds. Carbohydrates fermented with the production of H_2, CO_2 and 2,3-butylene glycol. Methyl red negative. Slow or no fermentation of lactose. The majority of species thus far described are from water or are known to be pathogenic to marine and fresh-water animals such as fish and amphibia".

The *Manual* lists four species, viz. *Aer. liquefaciens*, *Aer. punctata*, *Aer. hydrophila* and *Aer. salmonicida*.

Strains of bacteria which are now considered to belong to the genus *Aeromonas* have been isolated and described in greater or less detail during the past 70 years. They were all given different names and in their subsequent careers there were several converging lines which have met only in the last decade. These types will be dealt with individually in this section under the names originally assigned to them. The order in which they are treated is deliberately not chronological as it constitutes an attempt to deal in a systematic manner with each converging line.

Aerobacter liquefaciens *Beijerinck*

Beijerinck (1900), when investigating the production of hydrogen sulphide in the ditches of Delft, isolated several different types of fermentative bacteria, none of them producing diastase, for which he proposed the generic name *Aerobacter*, the type species being *A. aerogenes*. One of the species characterized by its rapid liquefaction of gelatin and by having a single, polar flagellum, was described by Beijerinck in the following way: "This species rapidly liquefies both wort and animal gelatin, and is one of the most potent fermentative agents of malt wort which there are. It is a rapidly motile, short rod with a polar flagellum which is difficult to stain. It occurs somewhat rarely in the mud of the ditches, usually in special, marshy places, and was found by me in the following way. Dried rhizome of *Althaea officinalis*, bought at the chemists, was cut into pieces, covered with water in a test-tube and left at 28°C for 1–2 days. A thick slime formed which developed vigorous gas production and yielded several varieties of *A. aerogenes*, but specially of *A. liquefaciens*, by streaking on wort gelatin. The fermentative powers are the same as those of *A. aerogenes*."

A few years later Harden & Walpole (1906), unable to account for all the glucose fermented by *Bacillus lactis aerogenes* (=*A. aerogenes*) in terms of acids produced, discovered that as much as one-third of the glucose was converted into 2:3-butanediol, accompanied by smaller amounts of acetylmethylcarbinol. Harden (1906) also showed that the presence of acetylmethylcarbinol is responsible for the positive reaction in the Voges-Proskauer test. It is oxidized by air in alkaline solution to diacetyl which gives a red colour with medium constituents (or added creatine) containing guanidine residues: 2:3-butanediol does not give this reaction.

In their classification of bacteria, Kluyver & van Niel (1936) proposed the name *Aeromonas* for polarly flagellated bacteria which ferment sugars with the production of acid and gas in a manner probably closely related to the fermentation type characteristic of the genera *Aerobacter* and *Aerobacillus*. Their description of the genus was as follows: "Short, rod-shaped bacteria, motile by means of cephalotrichous flagella. No endospores formed. Chemo-heterotrophic, oxidizing various organic compounds, and capable of fermenting carbohydrates with the production of carbon dioxide and hydrogen, and 2:3-butylene glycol (?). Gram-negative. The type species is *Aeromonas liquefaciens* Beijerinck. Syn: *Aerobacter liquefaciens* Beijerinck." Their hesitation over the specification of 2:3-butylene glycol (2:3-butanediol) was probably because (a) Beijerinck (1900) had said that *A. liquefaciens* had a fermentation pattern typical of the genus, while (b) Harden & Walpole (1906) had only later shown that the characteristic product of *Aerobacter* fermentation is 2:3-butanediol. In the absence of Beijerinck's original strain, its production of 2:3-butanediol could not be confirmed.

As *Aeromonas liquefaciens* this species is included, for the first time, in the 7th edition (1957) of Bergey's *Manual.*

Pseudomonas fermentans *von Wolzogen Kühr*

The negative correlation between the methyl red and Voges-Proskauer tests among coli-aerogenes bacteria had recently been demonstrated and von Wolzogen Kühr (1932) wished to test this correlation on fermentative organisms isolated from the faeces of *Chironomus plumosus* larvae. Among those he isolated were fermentative bacteria possessing one or more polar flagella. He named these organisms *Pseudomonas fermentans*, realizing that the polar flagellation differentiated them from the coli-aerogenes group. The organism was described in the 4th edition (1934) of Bergey's *Manual* under the name *Flavobacterium fermentans* but reverted to the original name in the 5th and 6th editions (1939, 1948). It is excluded from the 7th edition, one of von Wolzogen Kühr's original strains now being known as *Aeromonas liquefaciens* (L 417: Miles & Miles, 1951).

Bacillus hydrophilus fuscus *Sanarelli*

The earliest strains of this type, isolated from diseased frogs, were only sketchily described, the authors being more concerned with their pathogenicity. The first to be described was isolated by Ernst (1890) and named *Bacillus ranicida* but almost the only biochemical information recorded is that it liquefied gelatin rapidly. Growth on potato was light brown in colour.

Sanarelli (1891) described in somewhat more detail *B. hydrophilus fuscus* which he first isolated from the lymph and blood of frogs which died before they could be used in laboratory experiments: it was subsequently isolated from the laboratory water supply. This strain resembled Ernst's *B. ranicida* in liquefying gelatin and in giving a yellow to brown colouration on potato. In addition it liquefied coagulated serum and produced gas in glycerol agar. Because of the apparently differing temperature relations and pathogenicity, however, Sanarelli did not consider his strain to be identical with *B. ranicida*.

Roger (1893) isolated a very similar organism from dead frogs, from the aquarium water and from the water supply, and Russell (1898) isolated *B. hydrophilus fuscus* from a fatal disease in frogs occurring in epizootic form and characterized by a distinct reddening of the legs. He demonstrated its monotrichous flagellation. Again, in 1905 Emerson & Norris isolated an organism resembling Sanarelli's type and corresponding to Russell's description. They considered it to be more closely related to *B. hydrophilus fuscus* than to *B. ranicida*.

Chester (1897) renamed the organism *Bacterium hydrophilus fuscus* but in 1901 returned to the original generic name, at the same time reducing the binomial specific epithet to *hydrophilus* alone. In the 1st edition (1923) of Bergey's *Manual* the organism was described, under the name of *Proteus hydrophilus*, as having peritrichous flagella, though it had previously been shown to have polar flagella (Russell, 1898). This error remained until the 6th edition when the organism was described as being monotrichous and placed in the genus *Pseudomonas*, presumably after Kulp & Borden (1942) had re-investigated *Proteus hydrophilus* in some detail and re-established its monotrichous flagellation.

The polar flagellation was confirmed by Guthrie & Hitchner (1943). From frogs with 'red leg' they isolated organisms, differing significantly from typical *Proteus* (monotrichous flagellation, no swarming, urease-negative and producing acid and gas from mannitol), which they compared with strains of *Proteus hydrophilus* obtained from other sources. None of the strains differed significantly from descriptions of *Pseudomonas* (*Aeromonas*) *punctata*. Guthrie & Hitchner considered that organisms causing 'red leg' should be excluded from the genus *Proteus* and reclassified, and Stanier (1943) pointed out that the appropriate genus was *Aeromonas* Kluyver & van Niel and that *Proteus hydrophilus* might well be synonymous with *Aeromonas liquefaciens*, *Pseudomonas punctata* and *Pseudomonas fermentans*.

Proteus melanovogenes *Miles & Halnan*

Aitken, Barling & Miles (1936) isolated a non-lactose-fermenting, Gram-negative, gelatin-liquefying organism from a meat pie implicated in a fatal case of botulism. They considered the organism to be an atypical *Proteus*.

Miles & Halnan (1937) isolated several strains of an essentially similar type from eggs with black rot and established them as the main cause of the spoilage. They developed a method of isolating the organism and subsequently found strains in road dust, farmyard manure, chalk soil and human faeces (the only abnormal organism from a case of chronic colitis). They were unable to isolate the organism from hen faeces. Miles & Halnan recognized the resemblance of their strains to *Proteus hydrophilus* and also their differences from the type species *P. vulgaris*. Because of the mistaken description in Bergey's *Manual* of the flagella of *P. hydrophilus* as peritrichous, Miles & Halnan created a new species, *P. melanovogenes*, for their monotrichous strains. Haines (1938) compared one of Miles & Halnan's strains with *Proteus* strains producing black rot and found it to be biochemically distinct.

Following the establishment of *Proteus hydrophilus* as monotrichous and its transference to the genus *Pseudomonas*, Miles & Miles (1951) reinvestigated the surviving strains of *Proteus melanovogenes* and compared them with strains designated *Proteus hydrophilus*, *Pseudomonas ichthyosmia*, *Aeromonas liquefaciens* and *Aeromonas hydrophila* and with strains isolated from frogs with red-leg. Their results confirmed the conclusions drawn by Stanier (1943) and also established that *Proteus melanovogenes* is synonymous with *P. hydrophilus*. They put forward the opinion that the genus *Aeromonas* Kluyver & van Niel should be accepted if the production of 2:3-butanediol is shown to be characteristic of an otherwise substantially homogeneous group of cephalotrichous bacteria.

Bacillus(?) ichthyosmius *Hammer*

From a can of evaporated milk with a fishy smell, Hammer (1917) isolated a Gram-negative, fermentative organism which produced indole, digested casein and liquefied gelatin rapidly. This organism he named *B. ichthyosmius* though he was of the opinion that it was closely related to the *Proteus* group. In the 1st edition (1923) of Bergey's *Manual* this organism was described under the name of *Escherichia ichthyosmia*, and in the 4th as *Proteus ichthyosmius*. Breed, when revising the genus *Pseudomonas* for the 6th edition, established the monotrichous character of the organism and included it as *Ps. ichthyosmia*; it is absent from the 7th edition.

Bacillus punctatus *Zimmerman*

This organism was first isolated from Chemnitz tap water by Zimmerman (1890) and was subsequently found to be common in the River Main (Lehmann & Neumann, 1896). It differs very little from those already mentioned. In the first five editions of Bergey's *Manual* the organism was known as *Achromobacter punctatum*, but in the 6th edition it became *Pseudomonas punctata* following the work of Schäperclaus (1930, 1942). On the basis mainly of differing pathogenicity to fish, Schäperclaus distinguished five biological forms of *Ps. punctata*: f. *typica*, comparatively nonpathogenic; f. *Plehniae*, the cause of red pestilence in Westphalia; f. *sacrowiensis*, the cause of red boil disease and red pestilence in the Rivers Havel and Elbe; f. *ascitae*, the cause of ascites in members of the Cyprinidae, particularly carp, and common in North Germany; and f. *pellis*, probably the cause of the spotted disease of white fish. *Ps. punctata* f. *typica* and f. *ascitae* are indistinguishable physiologically; f. *Plehniae* is described as being Gram-positive and its inclusion in *Aeromonas* is therefore questionable.

Dombrowski (1953) and Wunder & Dombrowski (1953) recorded differences among strains of *Ps. punctata* in their action on milk, nitrate, blood and carbohydrates and in their production of indole and hydrogen sulphide. Schäperclaus (1958) attributed these results to the use of impure cultures.

Vibrio jamaicensis *Caselitz*

Hill, Caselitz & Moody (1954) isolated a monotrichous, Gram-negative, fermentative organism from a case of acute metastatic myositis and showed that it was capable of producing similar symptoms in mammals and birds. Because of the slight curvature of the cells they believed this organism to belong to the genus *Vibrio*. It was described in detail by Caselitz (1955) who proposed the name *Vibrio jamaicensis*. A similar organism was isolated from another patient, from tissues removed during an operation for abdominal actinomycosis complicated by idiopathic gangrene of the scrotum (Bras, Clearkin, Annamunthodo & Caselitz, 1954). These strains were subsequently compared with others isolated from human and animal sources (Caselitz, 1956), and the systematic position of these bacteria was further considered by Caselitz (1958). He concluded that *Vibrio jamaicensis* and related organisms should be included in the genus *Aeromonas*. Other similar strains from human sources have been described by Kjems (1955) and one by Caselitz, Hofmann & Martinez-Silva (1957–58) from osteomyelitis. Caselitz & Buck (1958) reviewed the characters of these bacteria and decided that they belonged in the genus *Aeromonas*.

Bacterium salmonicida *Lehmann & Neumann*

This organism was isolated by Emmerich & Weibel (1894); but most of their description is occupied by its appearance and action on gelatin, which they regarded as characteristic. The line of the stab became filled with air bubbles which on further liquefaction left sinuations on the gelatin corresponding to the partitions between them. Subsequently proving to be even more characteristic were the lack of motility and the production on agar of a diffusible brown pigment.

Marsh (1902) isolated a similar nonmotile, pigment-producing organism from diseased trout which he named *Bacterium truttae*. It was inactive towards glucose, lactose and sucrose and did not produce either indole or diastase.

In 1911 there was an epidemic in England, salmon and trout being killed in great numbers in the Rivers Exe, Dart and Teign, and other fish also in the Wye. Arkwright (1912) isolated nonmotile, Gram-negative organisms which closely resembled those of Emmerich & Weibel and which were fermentative.

Similar organisms were subsequently isolated in Scotland (Horne, 1928) and in 1929 the Minister of Agriculture and Fisheries set up the Furunculosis Committee to report on the disease. The bacteriological findings reported by the Committee (Report, 1930) show that apart from the pigment produced on agar and the lack of motility, *Bact. salmonicida* closely resembles other bacteria now included in *Aeromonas*. However, the genus *Aeromonas* was created for cephalotrichous bacteria having a fermentation pattern similar to that of *Aerobacter*. *Bact. salmonicida* is nonmotile and Voges-Proskauer negative; hence its inclusion in the genus *Aeromonas* in the 7th edition of Bergey's *Manual* is based on circumstantial rather than direct evidence.

The literature on *Aeromonas salmonicida* and its relation to furunculosis is reviewed by McCraw (1952) and Griffin (1954).

Aeromonas margarita *Stevenson*

From laboratory stocks of the desert locust infected by an epizootic disease, Stevenson (1959) isolated a fermentative bacterium, with a single polar flagellum, which he named *Aeromonas margarita*. Because of biochemical similarities between his strains and strains of *Serratia*, and because of the changes which have been shown to occur in the flagellation of *Aeromonas* (Leifson & Hugh, 1953) Stevenson suggested that his strains are, in fact, nonpigmented strains of *Serratia marcescens* and that all members of the genus *Aeromonas* are nonpigmented members of the genus *Serratia*.

(b) *The genus* Kluyvera

The genus *Kluyvera* was created by Asai, Okumura & Tsunoda (1956, 1957) for polarly flagellated organisms which carry out a mixed acid fermentation rather than a 2:3-butanediol fermentation, i.e. which bear the same relation to *Aeromonas* as *Escherichia* does to *Aerobacter*. The definition of the genus is as follows:—

"Gram-negative short rods. Motile with polar flagella. Ferment glucose and lactose with acid and gas (hydrogen present). Nitrites are produced from nitrates. Acetylmethylcarbinol is not produced. Methyl red test is positive. Milk is usually coagulated, but peptonization does not occur. Gelatin is not liquefied. Grow well on ordinary media and pigment is not produced. Found in soil and sewage."

Kluyvera citrophila *and* K. noncitrophila *Asai*

These organisms were isolated from soil and sewage in Japan and their most characteristic feature, apart from polar flagellation and fermentative ability, was their accumulation from glucose of large quantities of α-ketoglutaric acid (Asai, Okumura & Tsunoda, 1956, 1957). The characteristics of these organisms were studied in detail (Asai *et al.* 1957) and it seems clear that they are well differentiated from the *Aeromonas* types so far described. Two species were distinguished on the basis of ability to utilize citrate. Asai and his co-workers suggested that *Pseudomonas coli communis* and *Ps. formicans* probably also belong to *Kluyvera*.

Pseudomonas coli communis *Conn, Esten & Stocking*

This organism was isolated by Conn *et al.* (1906) from cheese. It fermented glucose with gas production, did not liquefy gelatin or digest casein and possessed a single flagellum.

Pseudomonas formicans *Crawford*

During an investigation of sewage for coli-aerogenes bacteria Crawford (1954) isolated a monotrichous, anaerogenic, fermentative bacterium which resembled *Escherichia coli* in being methyl red positive and Voges-Proskauer negative. However, strains of *Aerobacter* are known with these characters atypically reversed, and the utilization of citrate is not invariably positive: hence the data presented by Crawford are not sufficient to exclude this organism from the genus *Aeromonas*, and Pivnick & Sabina (1957) have suggested that it should for the time being be included in *Aeromonas*. They isolated 14 strains, from spent machine shop oil emulsions, which they compared with strains of Crawford's *Ps. formicans*. The only striking difference found lay in the ability of 13 of their 14 strains to utilize citrate. These organisms differ from *Kluyvera* in their lack of gas production and in other biochemical characters (see later). *Pseudomonas coli communis* seems to be more nearly related to *Kluyvera* than to *Pseudomonas formicans*.

(c) *The comparative morphology and flagellation of* Aeromonas *and* Kluyvera

According to Kluyver & van Niel (1936) the genus *Aeromonas* should consist of short, rod-shaped bacteria, motile by means of cephalotrichous flagella, and this description fits all the types of *Aeromonas* described hitherto with the exception of *Aer. salmonicida*, which is nonmotile The quoted cell sizes vary from 0·4–1·0 μ × 0·8–6·0 μ (6 μ is exceptionally long: cells rarely exceed 3–3·5 μ in length). Buttiaux (1959) shows an electron micrograph of an organism considered by him to be an *Aeromonas* but which is coccoid. Pleomorphism has been reported for *Aeromonas* and the cells may be capsulated (Kulp & Borden, 1942).

The genus is cephalotrichate and, in most descriptions, has one flagellum. Miles & Miles (1951), however, recorded two strains with from two to eight flagella. The coccoid *Aeromonas* illustrated by Buttiaux has seven flagella.

Leifson & Hugh (1953) showed that lateral flagella were present on *Aeromonas* cells during the early stages of growth. The period during which lateral flagella could be demonstrated decreased as the incubation temperature was raised. Strain L 418 was peritrichously flagellated through its life cycle. Leifson & Hugh suggested that their strain L 418 was a peritrichous variant, but biochemical evidence suggests that the strains examined by them and by the present author (see later) are not *Aeromonas*.

The strains of *Kluyvera* described have all been cephalotrichous with one, occasionally two or three, flagella.

(d) *The comparative biochemistry of* Aeromonas *and* Kluyvera

Descriptions of those organisms isolated before about 1930 are meagre and ideas have changed about which biochemical tests are useful in the systematic classifications of the various groups of bacteria. To estimate the constancy of biochemical behaviour in *Aeromonas* it is therefore necessary to confine attention to more recently isolated strains: with *Kluyvera* the problem does not arise. For this reason it is proposed to restrict biochemical discussion to the strains of *Aeromonas* discussed by

Miles & Miles (1951), Kjems (1955) and Hill *et al.* (1954), to *Aeromonas salmonicida* and to the strains of *Kluyvera* discussed by Crawford (1954), Pivnick & Sabina (1957) and *Asai et al.* (1957).

Table 1. *A comparison of the reported characters of some strains of* Aeromonas *and* Kluyvera *with those diagnostic for the coli-aerogenes group of the Enterobacteriaceae* (Report, 1958)

Character	Reactions† of Enterobacteriaceae genus						Reactions† of *Aeromonas*				Reactions† of *Kluyvera*		
	Citrobacter	*Escherichia*	*Klebsiella*	*Aerobacter*	*Hafnia*		Miles & Miles (1951)	Kjems (1955)	*Vibrio jamaicensis*	*Aer. salmonicida*	Asai *et al.* (1957)	Crawford (1954)	Pivnick & Sabina (1957)
					37°	22°							
Gas from glucose	+	+	+	+	d	+	+	+	+	+	+	—	—
Lactose	+/*	+/*	+	+/*	—/*	—/*	d	—/*	?	—	+	+	+/*
Sucrose	d	d	+	+	*	*	+	+	+	—	d	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	n.t.	+
Dulcitol	d	d	d	d	—	—	—	—	—	—	d	n.t.	n.t.
Salicin	d	d	+	+	d	d	d	d	—	n.t.	+	n.t.	n.t.
Adonitol	—	—	+	d	—	—	n.t.	—	n.t.	n.t.	n.t.	n.t.	n.t.
Inositol	—/*	—	+	d	—	—	—‡	—	—	n.t.	n.t.	n.t.	n.t.
Indole	—	+	—	—	—	—	+	+	+	—	d	+	d
Methyl red	+	+	—	—	+	—	d	n.t.	—		+	+	+
V-P§	—	—	+	+	d	+	+	+	+	—	—	—	—
Citrate	+	—	+	+	d	+	d	d	+	n.t.	d	—	d
H_2S	+	—	—	—	—	(+)	+	+	+	n.t.	+	n.t.	?
Urease	—	—	(+)	d	—	—	—	d	—	n.t.	—	n.t.	n.t.
Gelatin	—	—	—	d	—	—	+	+	+	+	—	n.t.	+
Cyanide	+	—	+	+	+	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Motility	+	+	—	+	d	+	+	+	+	—	+	+	+
Phenylalanine deaminase	—	—	—	—	—	—	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Malonate	—	—	+	d	d	d	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

† Reactions: +, positive 1–2 days; *, late and irregularly positive; —, negative; ?, doubtful; (+), urea hydrolyzed slowly, H_2S produced in small amounts; n.t., not tested; d, different biochemical types. ‡ One strain positive (L 418). § Voges-Proskauer.

Since the main character which distinguishes these organisms from members of the Enterobacteriaceae is flagellation, it is perhaps not inappropriate to think first of *Aeromonas* and *Kluyvera* in terms of those biochemical tests which have been found useful in differentiating the peritrichous, fermentative groups—in particular the coli-aerogenes and paracolon groups (Report, 1958).

The relevant characters are set out in Table 1. It is clear that *Aeromonas* and *Kluyvera* are types which do not fall into any of the genera of the Enterobacteriaceae. More work remains to be done before either *Aeromonas* or *Kluyvera* can be shown to be a genus with certain well defined and constant biochemical characteristics. Certain features, however, merit attention at this time. With the exception of *Aer. salmonicida*, *Aeromonas* seems to be consistently indole positive. This is noteworthy because it is coupled with a positive Voges-Proskauer reaction, a combination met with in the Enterobacteriaceae only in *Klebsiella aerogenes* II (Report, 1956). Unlike *Aeromonas*, however, such strains never liquefy gelatin rapidly.

Other characters which seem to be consistent in *Aeromonas* are: β-haemolysis of blood, peptonization of milk and particularly rapid liquefaction of gelatin.

2:3-*Butanediol fermentation and the Voges-Proskauer reaction.* Harden & Walpole (1906) showed that the main fermentation product of *Aerobacter* was 2:3-butanediol, smaller amounts of acetylmethylcarbinol also being formed, and Harden (1906) showed that it was the acetylmethylcarbinol which was detected by the Voges-Proskauer reaction. 2:3-Butanediol does not give a positive V-P reaction, but it is the production of this substance which is held to characterize the genus *Aeromonas* (Kluyver & van Niel, 1936; Miles & Miles, 1951).

In the formation of 2:3-butanediol, two molecules of pyruvic acid condense with decarboxylation to give α-acetolactic acid which is then further decarboxylated to acetylmethylcarbinol. This is then reduced to 2:3-butanediol, the proportion of the two being determined by the redox potential. In *Aerobacter aerogenes* a positive V-P reaction is taken as sufficient indication of this type of behaviour, and in such quantitative investigations as have been made on the fermentation products of *Aeromonas* (Stanier & Adams, 1944; Murphy, Watson, Muirhead & Barnwell, 1951) 2:3-butanediol has almost always been found to be accompanied by acetylmethylcarbinol. Nevertheless, in one instance (Murphy *et al.* 1951) acetylmethylcarbinol was quantitatively converted into 2:3-butanediol, and V-P negative types ought therefore to be tested for the glycol. If they are still found to be negative, both their inclusion in the genus and the use of 2.3-butanediol production as a diagnostic character will need to be reconsidered.

The strain used by Stanier & Adams (1944) was probably not *Aeromonas* since it fermented xylose, which *Aeromonas* does not (see later), and the strains used by Murphy *et al.* (1951) have since been shown to be *Aerobacter cloacae* (Clement & Gibbons, 1960). Nevertheless, these new facts do not invalidate the suggestion that V-P negative cultures ought to be tested for the glycol.

The case of *Aeromonas salmonicida* deserves special attention, because besides being V-P negative it is also nonmotile.

Ps. formicans (Crawford, 1954; Pivnick & Sabina, 1957) differs from *Kluyvera* (Asai *et al.* 1957) in its failure to produce gas from glucose, its failure to ferment arabinose and xylose, and its ability to hydrolyze starch and gelatin. *Ps. coli communis* seems to be more closely related to *Kluyvera* than to *Ps. formicans*, since it produces gas and does not liquefy gelatin.

(e) *The temperature relations of* Aeromonas *and* Kluyvera

In view of the wide range of habitats from which these organisms have been isolated—water, diseased fish and amphibia, and man—it is perhaps not surprising that they show a correspondingly wide range of behaviour in response to temperature. The strains which have been described can be grouped into three classes according to whether the optimum temperature is about or below 20°, is between 25 and 30° or is near 37°.

(i). The only type which might be termed genuinely psychrophilic (*sensu* Buchanan & Fulmer, 1930) is *Aer. salmonicida.* Emmerich & Weibel (1894) gave the optimum temperature as between 10 and 15° but later workers have found it to be nearer 20°. Arkwright (1912) specified 18–20° as the optimum temperature, growth being slow at +5° and poor at 36°, and the Furunculosis Committee (Report, 1930) gave an optimum of about 20° and a range of 5–32°. *B. truttae* (Marsh, 1902) showed similar behaviour. The optimum temperature of *B. ichthyosmius* was also given as 20°; Hammer (1917) found that more gas was produced from sugars at room temperature than at 37°.

(ii). *Aer. punctata* has an optimum temperature lying between 25 and 30° (Schäperclaus, 1930). Grown cultures transferred to 37° died, but some freshly inoculated cultures were able to grow after suffering initial damage. Ernst (1890) obtained good growth of *B. ranicida* at 22°, better growth at 30° and no growth at 8·5 or 38°.

Kluyvera has an optimum temperature of 30° (Asai *et al.* 1957), and does not grow at 42°. *Ps. formicans* grows best at 30° but good growth is obtained between 18 and 42°.

(iii). The remaining organisms fall into two categories according to whether or not they were obtained from human sources. *Aer. hydrophila* has an optimum temperature of 37° (Russell, 1898; Emerson & Norris, 1905) as have also *Ps. fermentans* (von Wolzogen Kühr, 1932) and *Proteus melanovogenes* (Miles & Halnan, 1937). These organisms were isolated from low temperature habitats. The optimum temperatures of the human strains have not been investigated but biochemical tests have been made on them at 37° (Hill, Caselitz & Moody, 1954; Kjems, 1955).

It would clearly be of interest to know the lower limit of temperature which will permit the growth of these organisms. Eddy & Kitchell (1959) recorded a strain of *Aeromonas* with an optimum temperature of 37° which grew well at +1·5°.

(f) *The pathogenicity of* Aeromonas

The pathogenicity of these organisms would be expected to bear a relationship to their reactions to temperature; an organism with a low optimum temperature is unlikely to be pathogenic for warm-blooded animals. *Aer. punctata* and *Aer. salmonicida* have only been shown to be pathogenic for fish (Schäperclaus, 1930, 1942; Report, 1930). No active toxin production by *Aer. salmonicida* has been demonstrated and it has been suggested that its pathogenicity may depend upon an interference with the blood supply to tissues and organs associated with their necrosis and destruction.

Ernst (1890) reported that *B. ranicida* was pathogenic only for cold-blooded animals but Sanarelli (1891) found *B. hydrophilus fuscus*, a very similar organism, to be pathogenic for both cold- and warm-blooded animals, findings which were confirmed by Russell (1898) and Emerson & Norris (1905) though there are variations in detail. Sanarelli found his organism to be pathogenic for frogs, toads, salamanders and lizards (barbels and freshwater eels were more resistant), and for hens, pigeons, mice, bats, guinea pigs, hedgehogs, rabbits and newly-born puppies and kittens. With dogs he observed no effect and with cats only lesions near the point of injection which healed slowly and with difficulty. The failure with the larger animals may have been a question of dose. Russell's results differed from those of Sanarelli only in that pigeons exhibited merely a transient sickness. Emerson & Norris found that their strain was not pathogenic for rabbits.

Russell (1898) found a culture filtrate of *B. hydrophilus fuscus* to be without effect on frogs but, following Trambusti (1893), he showed that the solids of the culture filtrate contained powerful toxins; one was soluble in alcohol and the other insoluble, and they had different physiological actions on the heart and muscle of frogs.

Roger (1893) showed that *B. hydrophilus fuscus* was also pathogenic for carp and, arguing from its demonstrated pathogenicity for warm-blooded animals, questioned whether it could not produce, directly or indirectly, infection in man.

Miles & Halnan (1937) showed that their strains of *Proteus melanovogenes* would cause the death from acute septicaemia of mice and guinea-pigs within 24 hr of intraperitoneal injection. Culture filtrates were toxic to mice.

The first organism to be isolated from a human source was found by Miles & Halnan (1937) in faeces from a case of chronic colitis, but no causal relationship was demonstrated. Hill, Caselitz & Moody (1954), however, isolated *Vibrio jamaicensis* from a case of metastatic myositis and showed that the organism produced the same symptoms in rabbits, other animals and pigeons. Other similar types isolated have been associated with gangrene (Bras *et al.* 1954), septicaemia (Kjems, 1955) and osteomyelitis (Caselitz, Hofmann & Martinez-Silva, 1957-8).

Ps. fermentans and *Ps. ichthyosmia* have never been associated with any pathological condition, nor have any of the strains classified as *Kluyvera* by Asai *et al.* (1957).

(g) *The serology of* Aeromonas

Miles & Halnan (1937) prepared an antiserum against one of their strains of *Proteus melanovogenes* and determined with it the agglutination titres of their other strains, both with the flagella intact and after the flagella had been destroyed by alcohol. They concluded that their strains, though resembling each other biochemically, were antigenically heterogeneous, and that most of the agglutinin response was due to flagellar antigens.

Guthrie & Hitchner (1943) compared strains isolated from frogs suffering from red-leg with strains of *Proteus hydrophilus* and found that they constituted an antigenically heterogeneous group.

Antigenic heterogeneity was also found by Miles & Miles (1951) when testing twelve strains from different sources against antisera produced from seven of them. Some close relationships were found but, in general, agglutinating titres with different strains were small compared with homologous titres.

Kjems (1955) carried out cross agglutination experiments with five strains, using antigens produced in different ways. Two strains had related O antigens but the strains were otherwise unrelated.

Caselitz & Schönn (1956) tested *Vibrio jamaicensis* against eight related strains. Four of the nine (V.j., PM 25, PM 68 and C 361) showed clear antigenic relationships, V.j. and PM 68 having the same O antigen, and PM 25 and C 361 also having similar antigenic components. Strain C 173 seemed in addition to have a somatic surface antigen. After 2 hr heat treatment all the strains agglutinated in the available O-sera. Five strains of V.j. and 16 *Vibrio* strains from water reacted only after intensive heat treatment. Weak cross agglutination reactions with haemolytic *Escherichia coli* and *Proteus* strains were demonstrated after heating for 2 hr. Biochemically, strains V.j., PM 25, PM 68 and C 361 differed only in their ability to produce indole, V.j. and PM 68 being positive and PM 25 and C 361 negative (Caselitz, 1956).

Caselitz & Buck (1958) prepared antisera against three strains from water in Korea and one strain from a case of osteomyelitis, and tested them against the same strains and against two others from Korea and two strains of *Ps. punctata*. Two of the Korean strains and that from osteomyelitis showed common antigenic components, but no other relationships could be demonstrated.

In view of the degree of antigenic heterogeneity exhibited by the strains of *Aeromonas* tested, it seems unlikely that much would be achieved by testing them against other genera. Nevertheless, Caselitz & Schönn (1956) did obtain weak cross agglutinations with *E. coli* and *Proteus*. It would indeed be interesting if it could be shown whether *Aeromonas* is more closely related antigenically to the oxidative, cephalotrichous Pseudomonadaceae or to the fermentative, peritrichous Enterobacteriaceae.

4. A Comparative Study of Presumptive *Aeromonas* and *Kluyvera* Organisms

In this section are described the results of a comparative study of 54 strains of bacteria presumed to be members of the genera *Aeromonas* and *Kluyvera*. Some of the strains were named species and others were unnamed strains isolated from various sources in this laboratory or elsewhere. All four species of *Aeromonas* listed in Bergey's *Manual* (7th ed.) and both species of *Kluyvera* are represented, as well as two species of *Photobacterium* (Spencer, 1955). Table 2 lists the designations and sources of the strains investigated.

(a) *Methods*

For primary isolation the following procedure proved satisfactory although, for reasons which will appear later, it is more applicable to Voges-Proskauer positive than to V-P negative strains. The sample was plated out on peptone water agar containing 5% of oxalated horse blood and 30% of dialyzed skimmed milk. After incubation at 30° for 48 hr, colonies which were both haemolytic and caseinolytic were tested for oxidase by Kovacs' (1956) method. Positive strains were then tested for ability to ferment glucose either by acid and gas production in glucose peptone water or, for anaerogenic strains, by acid production using the anaerobic technique devised by Hugh & Leifson (1953). There is a strong presumption that fermentative organisms will belong to this group: confirmation is obtained by demonstrating polar flagellation.

In carrying out the tests listed below, incubation was at 30° unless otherwise stated. *Aeromonas salmonicida* strains 161–164 did not grow well at 30° so the tests were carried out at 25°. The strains of *Photobacterium* were tested in media containing 3% of NaCl.

Morphology, motility and flagellation. Morphology was studied using preparations from agar cultures stained by Gram's method. Motility was determined by microscopic examination of a hanging drop of a 24 hr peptone water culture. For the demonstration of flagellation the organisms were grown overnight on a nutrient digest agar slope to which 2 ml of sterile water had been added, and the flagella were stained by Rhodes' (1958) modification of Fontana's silver-plating method for spirochaetes. Preparations for photomicrography were made by gold-palladium shadowing and by negative staining with phosphotungstic acid (M. J. Thornley, pers. comm.).

Table 2. *The designations and sources of the strains of* Aeromonas *and* Kluyvera *investigated*

Strain	Designation and source*	
16	*Pseudomonas hydrophila* NCTC 7810	
17	*Ps. hydrophila* NCTC 7812	
18	*Ps. formicans* (Crawford, 1954)	
95	*Aeromonas* from eviscerated poultry stored at +3° (M. J. Thornley) (Eddy & Kitchell, 1959)	
96	*Aer. liquefaciens* L417 (=*Ps. fermentans*)	(Miles & Miles, 1951)
97	*Aer. liquefaciens* L 418	
98	*Aer. hydrophila* DF1a	
108	A polarly flagellated member of the Enterobacteriaceae (Cook, 1958)	
112–119	Artesian well water (E. M. Barnes)	
138	*Aer. hydrophila* CCEB 388	
139	*Aer. liquefaciens* CCEB 521	
140	*Aer. punctata* CCEB 386	
141	*Aer. salmonicida* CCEB 382	
142	*Cloaca cloaca* NCTC 9744	
143	*Ps. ichthyosmia* NCTC 8049	
144	*Aeromonas* B2	(Caselitz, 1955; Caselitz & Buck, 1958)
145	*Aeromonas* BD	
146	*Aeromonas* BE	
147	*Aeromonas* VT1	
148	*Vibrio jamaicensis*	
149	*Vibrio jamaicensis* Prof. K. R. Hill[1]	
150	*Aeromonas* E.L.	Dr. E. Kjems[2]
151	*Aeromonas* 3028	
152	*Aeromonas* 455	
153	*Aeromonas* A.19	
154	*Aeromonas* A.20	
155	From a mixture of goldfish faeces and uneaten fish food	
156	From human faeces	
157	*Aeromonas* O 27	Dr. R. Buttiaux[3]
158	*Aeromonas* O 38	
159	*Aeromonas* L 2	
160	*Aeromonas* E 72	
161	*Aer. salmonicida* 1046	Dr. Isabel Smith[4]
162	*Aer. salmonicida* 1047	
163	*Aer. salmonicida* 3763	
164	*Aer. salmonicida* 3896	
165	*Aeromonas* G 1 Mr. W. Hodgkiss[5]	
166–167	*Kluyvera citrophila* α	Prof. T. Asai[6]
168–169	*Kluyvera noncitrophila*	
170	*Ps. punctata* f. typica 29	Prof. W. Schäperclaus[7]
171	*Ps. punctata* f. typica 43	
172	*Aeromonas* 125	Prof. A. Wilssens[8]
173	*Aeromonas* 214	
174	*Photobacterium harveyi*	Dr. R. Spencer[9] (Spencer, 1955)
175	*Photobacterium sepia*	

* NCTC, National Collection of Type Cultures; CCEB, Culture Collection of Entomogenous Bacteria, Prague.

[1] The Royal Free Hospital, London. [2] Statens Seruminstitut, Copenhagen. [3] Institut Pasteur de Lille. [4] Scottish Home Dept., Marine Laboratory, Aberdeen. [5] Torry Research Station, Aberdeen. [6] Institute of Applied Microbiology, Tokyo. [7] DAL Institut für Fischerei, Berlin-Friedrichshagen. [8] Rijkslandbouwhogeschool, Gent. [9] Humber Laboratory, Hull.

Voges-Proskauer and methyl red reactions. These were tested after growth in glucose-phosphate-peptone water for 3 and 7 days. Acetylmethylcarbinol was detected by reddening following the addition to 1 ml of culture of 0·6 ml of 5% (w/v) α-naphthol in absolute alcohol and 0·2 ml of 40% (w/v) KOH containing 0·3% of creatine, and shaking. Where the MR reaction was doubtful the test was repeated using the medium of Fouad & Richards (1953) but no improvement resulted.

2:3-*Butanediol production.* V-P negative strains were grown in 50 ml of glucose (2·5%w/v)-phosphate-peptone water for 7 days. The cultures were neutralized, autoclaved and extracted with ether. The ether was evaporated and the residue taken up in 5 ml of water. The resulting solution was spotted on paper and sprayed with a 1% (w/v) solution of lead tetra-acetate in benzene (Buchanan, Dekker & Long, 1950). It was found that, even after drying over calcium chloride and distilling, the benzene contained sufficient moisture to decompose the reagent. This was overcome by adding 5% (v/v) of acetic anhydride to the benzene.

Fermentation tests and hydrolysis of glycosides. The ability of the organisms to ferment the following substances was tested, using peptone water cultures with Andrade's indicator and Durham's tubes: glucose, raffinose, cellobiose, maltose, lactose, sucrose, trehalose, fructose, galactose, mannose, rhamnose, arabinose, xylose, adonitol, dulcitol, inositol, mannitol, sorbitol, glycerol, dextrin, starch, inulin, glycogen and salicin. Ability to hydrolyze aesculin was tested by blackening following growth on peptone water agar containing 0·1% of aesculin and 0·1% of ferric chloride. Action on salicin and aesculin was examined at several temperatures.

Malonate utilization. This was tested by the method recommended in Report (1958).

Hydrogen sulphide production. This was detected by blackening in Kligler's iron agar slopes, in ferrous chloride-gelatin stabs (incubated at 20°) or of lead acetate papers suspended above broth cultures.

Reduction of nitrate. By testing for nitrite following growth in broth containing 1% (w/v) of potassium nitrate.

Indole production. This was tested for in peptone water cultures, after 3 and 7 days' incubation, with Ehrlich's reagent.

Utilization of citrate. By growth on Simmons' citrate agar slopes (Report, 1958).

Catalase. A loopful of a 24 hr agar culture was transferred to a drop of '10 vol.' hydrogen peroxide and watched for the production of gas bubbles.

Urease. By overnight growth on Christensen's urea agar slopes.

Phenylalanine deaminase. By growth on agar containing phenylalanine and subsequent testing with 10% (w/v) ferric chloride (Report, 1958).

Arginine test. Anaerobic production of ammonia from arginine was tested using the medium 2A of Thornley (1960).

Oxidase. By testing the growth on a 24 hr agar slope with 1% (w/v) tetramethyl-*p*-phenylenediamine dihydrochloride as described by Kovacs (1956).

Hydrolysis of gelatin. By using gelatin stabs incubated at 15°.

Hydrolysis of casein. By surface growth on peptone water agar plates containing 30% (v/v) of skimmed milk. For lactose fermenting strains the milk was first dialyzed, as it was found that the acid produced in lactose fermentation had an inhibitory effect on the hydrolysis of the casein.

Haemolysis. By growth on agar containing 5% (v/v) of oxalated horse blood. Haemolytic strains were tested for filtrable haemolysin by incubating washed horse red cells with membrane-filtered broth culture.

Liquefaction of serum. By growth on Löffler's serum slopes.

Growth in the presence of potassium cyanide. By use of the peptone medium containing $1{\cdot}1\times10^{-3}$ M KCN described in Report (1958).

Resistance to penicillin, terramycin and the vibriostat O/129. Streaks of peptone water cultures were made on nutrient agar plates and a tablet of penicillin (2·5 i.u.) and a tablet of terramycin (10 µg) were placed on top of each streak. Resistance to the vibriostat was tested by streaking an agar plate, making a hole with a small cork borer at a point on the streak and filling it with a saturated aqueous solution of the pteridine derivative O/129 (Shewan, Hodgkiss & Liston, 1954). The plates were then incubated and zones of inhibition of growth noted.

Production of black rot in hens' eggs. Pairs of eggs were inoculated with 0·5 ml of a broth culture diluted until it contained about 2×10^6 cells/ml, as described by Miles & Halnan (1937). The eggs were incubated, and candled after 7, 14 and 21 days. When after 3 weeks one or both of the eggs in any pair showed no rot two more eggs were inoculated with 0·5 ml of undiluted broth culture. For comparison the strains were grown on Dorset's egg slopes.

Growth at +1°. Loops of peptone water culture were streaked on the surface of nutrient agar plates which were inspected after 7 and 14 days' incubation at +1°.

Pathogenicity to goldfish and mice. A saline suspension of bacteria centrifuged from a 24 hr broth culture was injected intraperitoneally into single goldfish (0·2 ml) and pairs of mice (0·5 ml). The suspensions contained *c.* 4×10^9 cells/ml.

Growth and reaction on media diagnostic for coli-aerogenes bacteria. All strains were tested on the CNBLM medium of Mossel (1957). The lactose fermenting strains were tested on MacConkey's broth, MacConkey's agar (Oxoid No. 3), brilliant green-bile broth and desoxycholate-citrate agar. Incubation was at 37° for 24 hr and 30° for 48 hr in all cases.

(b) *Results*

The results of tests which have proved useful in classification are given in Table 3, with the 54 strains grouped accordingly. Groups I, III and V consist of polarly flagellated or nonmotile oxidase positive strains, and groups II (strains 97, 108, 158 and 160) and IV (strains 166–169) of peritrichous, oxidase negative organisms which are not considered to belong to *Aeromonas*. The results of the other tests, and general comments, are given below in the same order as they occur in the Methods section.

Morphology, motility and flagellation. All the organisms examined were Gram-negative, rod shaped bacteria. Morphologically the strains in groups I and III were very similar, measuring usually *c.* $0{\cdot}4 \times 1\text{–}1{\cdot}5\ \mu$, but often showing a pleomorphism exemplified by very long cells—strain 116 was particularly notable in this respect. The group V strains (*Aer. salmonicida*) were shorter and rather fatter rods, readily distinguishable from the strains of groups I and III.

The members of groups I and III all possessed a single, polar flagellum (Plate 1, a and b). Preparations of strain 150, made as described, persistently showed only peritrichate cells, though from the biochemical evidence this strain was clearly an *Aeromonas* sp. If the water containing the loops of culture was incubated at 37° for 6 hr instead of at 30° for 1 hr, however, preparations showed a large majority of cells with single, polar flagella (cf. Leifson & Hugh, 1953).

Strain 97 was peritrichous, in confirmation of the finding of Leifson & Hugh (1953), and strains 141 and 161–4 (*Aer. salmonicida*) were nonmotile (confirmed by growth in sloppy agar with a Craigie tube at various temperatures, viz. 25, 20, 15 and 10°). Strain 108, described by Cook (1958) as being polarly flagellated, was found to be peritrichous: most of the cells had a single flagellum, often arising laterally, and a few were multitrichous. Strain 160 was similar to strain 108 and it has not yet been possible to demonstrate the flagellation of strain 158. The strains of *Kluyvera* (166–9) were found to be peritrichous (Plate 1, c and d), thus contradicting the conclusions of Asai *et al.* (1957).

Voges-Proskauer and methyl red reactions. The results of the V-P tests are given in Table 3. Those obtained with strains 16 (NCTC 7810) and 143 (NCTC 8049) agree with those of Miles & Miles (1951) and conflict with those of Rhodes (1959). They were confirmed using the medium of Fouad & Richards (1953). With the V-P negative strains the MR reaction was positive. With the V-P positive strains the MR reaction varied from negative to positive, i.e. the negative correlation encountered in the coli-aerogenes group was not a constant feature (cf. Caselitz, 1958).

2:3-*Butanediol production.* Using strain 96 as a positive control, the V-P negative strains 18, 113, 140 and 141 were tested for the production of 2:3-butanediol. All four were negative with lead tetra-acetate, whereas strain 96 gave a positive reaction.

Fermentation tests and hydrolysis of glucosides. The results obtained with glucose, raffinose, sucrose, cellobiose, trehalose, mannose, arabinose, xylose, dextrin, starch and glycogen are shown in Table 3. All the strains fermented glucose and it should be noted that a plus sign in the Table indicates gas production: with the other substances a plus sign records fermentation irrespective of whether gas was produced or not. This method of presenting the results corresponds with that adopted in Report (1958). The rapid fermentation of starch is of interest since this is only very rarely found in the Enterobacteriaceae (M. J. Parker, pers. comm.).

All the strains fermented maltose, fructose, galactose (not strain 175) and mannitol and none fermented adonitol or inulin. Dulcitol was fermented only by the peritrichous strain 160, inositol only by the peritrichous strain 97 and the only strains which did not ferment glycerol were no. 175 and the peritrichous no. 168. The results with the other substances were variable: lactose was fermented by strains 95, 98, 108, 114, 116, 144, 145, 149, 150, 157–160, 166–169 and 172–174; rhamnose was fermented by strains 108, 115, 117, 138, 158, 160 and 165–169; sorbitol was fermented by strains 17, 95, 97, 98, 112, 114, 116, 152, 160, 170 and 173; salicin was fermented by all the strains except nos. 16, 96, 97, 139, 147–149 and 171; and aesculin was hydrolyzed by all the strains except nos. 16, 96, 97, 139, 147, 148 and 171.

Only strains 95, 98, 108, 160, 166, 167, 169 and 173 produced acid and gas from lactose (173 in 6 days): strains 114, 116, 155, 157–159, 168, 172 and 174 produced acid within 2–3 days and strains 144, 145, 149 and 150 produced acid in 6–7 days. The results with salicin were interesting, as Miles & Miles (1951) also found. Of the 46 positive strains, 26 produced acid and gas at 30° and 10 only acid. Ten of the positive strains failed to ferment salicin at 30° but, of these, four (140, 141, 152 and 173) produced acid and gas and two (119 and 143) only acid, at 20°: strains 161–164 produced acid and gas from salicin most rapidly at 15°. There was a strong positive correlation between the ability to ferment salicin and hydrolyze aesculin, but not between these and the fermentation of cellobiose, the other β-glucoside used. All the strains which fermented cellobiose also fermented salicin and hydrolyzed aesculin, but the reverse was not the case. The ability of some strains to ferment salicin at 15° or 20° but not at 25° or 30°, whereas aesculin is hydrolyzed at 25° or 30°, may be due to a toxic action of saligenin at the higher temperature (J. A. Barnett, pers. comm.).

Malonate utilization. None of the strains except 108, 158, 160 and 166–169 (groups II and IV) was able to utilize malonate (Table 3).

Hydrogen sulphide production. In Kligler's iron agar all the strains were negative, whereas lead acetate papers suspended over broth cultures showed that they were all positive except nos. 163 and 164. In ferrous chloride-gelatin stabs all except strains nos. 18, 97, 108, 113, 156–164, 166–169, 174 and 175 were positive (Table 3).

Table 3. *The results of tests yielding characters found useful in classification, with the strains grouped accordingly*

Test*	Characters† of group I strains, nos.														
	16	17	95	96	98	112	114	115	116	117	118	119	138	139	142
V-P*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flagellation	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Oxidase*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sucrose	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Cellobiose	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	—	+	+	+	+	+	+	+	+	+	—	+
Xylose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Dextrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malonate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H_2S*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+
Citrate	—	+	+	+	+	+	—	—	+	+	+	—	—	+	+
Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Haemolysin*	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+
Cyanide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Black rot	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+
Goldfish*	1	1	S	5	2	1	2	1	1	1	1	2	2	2	1

Test*	Characters† of group I strains, nos.															
	143	144	145	146	147	148	149	150	151	152	153	154	165	170	171	173
V-P*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flagellation	C	C	C	C	C	C	C	C‡	C	C	C	C	C	C	C	C
Oxidase*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	‡	+
Raffinose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sucrose	+	+	—	—	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	—	l	l	—	—	—	—	—	+	—	—	—	—	—	—	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	—	—	—	+	+	+	—	—	+	+	—	—
Xylose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Dextrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malonate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H_2S*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	l	l	—	—	+	+	+	+	—	+	+	+	—	+	—	+
Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	w	w	w	+	w	w	w	w	+	+	+	w
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Haemolysin*	+	+	+	+	—	+	+	+	+	—	+	+	+	+	—	—
Cyanide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Black rot	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Goldfish*	2	2	1	2	2	S	1	1	1	2	S	1	1	2	S	S

Table 3 (*contd.*). *The results of tests yielding characters found useful in classification, with the strains grouped accordingly*

	Characters† of strains of group																					
	II, nos.				III, nos.										IV, nos.				V, nos.			
	97	108	158	160	18	113	140	155	156	157	159	172	174	175	166	167	168	169	141	161	162	16[illegible]	164
V-P*	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Flagellation	P	P	?	P	C	C	C	C	C	C	C	C	C	C	P	P	P	P	O	O	O	O	O
Oxidase*	—	—	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	—	+	+	+	+	+
Gas from glucose	+	+	—	+	—	+	+	—	—	—	—	—	—	—	+	+	—	+	+	+	+	+	+
Raffinose	+	+	—	+	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	—	—	—	—	—
Cellobiose	—	+	+	+	+	—	+	+	—	+	+	+	+	+	+	+	+	+	—	—	—	—	—
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	l	l	—	—
Mannose	+	+	+	+	—	+	+	+	—	+	+	+	+	+	+	+	+	+	—	l	l	l	l
Arabinose	+	+	+	+	+	+	—	+	+	+	+	+	—	—	+	+	+	+	—	l	l	l	l
Xylose	+	+	+	+	—	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—
Dextrin	—	—	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	—	+	+	+	+	+
Starch	—	—	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	—	+	+	+	+	+
Glycogen	—	—	—	—	+	+	+	+	+	+	+	+	+	+	—	—	—	—	+	+	+	+	+
Malonate	—	+	+	+	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—
H_2S*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
Indole	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
Citrate	+	+	+	+	+	—	—	—	+	—	—	l	—	—	+	+	—	—	—	—	—	—	—
Arginine	—	—	—	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—	+	+	+	+	+
Gelatin	+	—	+	—	+	w	+	+	—	+	+	+	w	w	—	—	—	—	+	+	+	+	+
Casein	—	—	—	—	w	+	+	+	+	+	+	+	—	+	—	—	—	—	+	+	+	+	+
Haemolysin*	●	●	●	●	—	—	—	—	—	—	—	—	—	—	●	●	●	●	+	+	+	+	+
Cyanide	+	—	—	—	+	+	+	+	+	+	+	+	●	●	+	+	+	+	—	—	—	—	—
Black rot	—	—	—	—	+	?	+	+	—	+	+	+	—	—	●	●	●	●	—	—	—	—	—
Goldfish*	●	●	●	●	S	2	S	4	S	S	S	S	S	S	●	●	●	●	2	2	S	S	2

* Tests: V-P, Voges-Proskauer; oxidase, Kovacs; H_2S, in $FeCl_2$-gelatin; haemolysin, filtrable; goldfish, days to death.

† Characters: + positive; l, late; w, weak; —, negative; ?, doubtful; ●, not tested; C, cephalotrichous; P, peritrichous; O, non-motile; S, survived; ‡ After prolonged incubation at 37° (see text).

Reduction of nitrate. All the strains gave positive reactions for nitrite except no. 140 (*Aer. punctata*). This species is stated to be unable to reduce nitrate (Bergey's *Manual*, 1st ed.), but according to Schäperclaus (1958) positive strains do occur, and this is exemplified by strains 170 and 171, tested here. Some of the strains destroyed nitrite so rapidly that the test had to be carried out as soon after inoculation as the culture became visibly turbid, because after 24 hr it was negative.

Indole production. The results of tests for indole production are shown in Table 3. *Aer. salmonicida* (141 and 161–164) has previously been reported to be indole negative (Arkwright, 1912; Report, 1930) but the negative reaction obtained with strain 138 is exceptional for motile *Aeromonas*.

Utilization of citrate. The results of citrate utilization tests using Simmons' citrate agar slopes are shown in Table 3. The rapid, positive result with strain 150 differs from that obtained by Kjems (1955).

Catalase. All the strains were able to decompose hydrogen peroxide.

Urease. None of the strains was positive: these results with strains 108, 153 and 154 differ from those of Cook (1958) and Kjems (1955).

Phenylalanine deaminase. None of the strains gave a strongly positive reaction with $FeCl_3$, but thirteen of them gave a very weak reaction, a green tint being visible in the outer layer of the agar when viewed along the surface with strains 18, 108, 138, 141–143, 147–149, 153, 158, 160 and 173. The remaining strains were negative.

Arginine test. The results for this test are shown in Table 3. All the strains were positive except those in groups II and IV and nos. 174 and 175.

Oxidase. This test was devised by Kovacs (1956) for the identification of *Ps. aeruginosa* and the reaction has since been shown to be given by many saprophytic pseudomonads though not by some plant pathogenic species. It is also given by some *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Rhodopseudomonas*, *Rhodospirillum* and *Vibrio* spp., but by no members of the Enterobacteriaceae.

With the exception of the strains in groups II and IV, those under present investigation were positive (Table 3). Ewing & Johnson (1960) have also reported the differentiation of *Aeromonas* from the Enterobacteriaceae by means of an oxidase test.

Hydrolysis of gelatin. One of the earliest characteristics to be recorded constantly for strains now thought to belong to *Aeromonas* was the ability to liquefy gelatin rapidly. The results of the current investigation are shown in Table 3: with strains recorded as positive visible liquefaction was obtained within 2–3 days at 15°. Strains 147–149 and 151–154, recorded as weak in Table 3, were isolated from human sources; hence it was thought that the weak reaction at 15° may have been due to temperature. This was confirmed by the demonstration of rapid liquefaction by these strains at 25°.

Hydrolysis of casein. The results are shown in Table 3. The reaction of strain 95 at first seemed to be weak, but when dialyzed skimmed milk was used the reaction was strong. It seems that acid production from lactose, besides precipitating the casein in the medium, interferes in some way with the hydrolysis. The same phenomenon was not observed when litmus milk was used: the acid clot was rapidly peptonized.

Haemolysis. Zones of β-haemolysis of varying width were observed with all strains except nos. 108, 113, 158–160 and 166–169. When the positive strains were tested for filtrable haemolysin the results in Table 3 were obtained. According to Bergey's *Manual* (7th ed.), *Aer. liquefaciens* is non-haemolytic; but Miles & Miles (1951) found their strain L 417 (no. 96) to be positive and strain L 418 (no. 97) to be negative. The latter was not tested for filtrable haemolysin in the present investigation, but the former was, and was positive.

Liquefaction of serum. None of the strains caused complete liquefaction of coagulated serum. All except the strains in groups II and IV and nos. 113, 156, 174 and 175, however, produced clearing and varying degrees of softening.

Growth in the presence of potassium cyanide. Growth occurred with all the strains tested except nos. 108, 141, 158 and 160–164 (Table 3).

Resistance to penicillin, terramycin and the vibriostat. With a few exceptions, all the strains were sensitive to terramycin and resistant to penicillin and the pteridine derivative O/129. Strain 112 was insensitive to terramycin, strain 161 was sensitive to penicillin and strain 158 was weakly sensitive to the vibriostat. Rhodes (1959) found strains 16 and 143 (NCTC 7810 and 8049) to be sensitive to O/129, but that result was not obtained here.

Production of black rot in hens' eggs. The results are shown in Table 3. On Dorset's egg medium the strains which produced black rot in whole eggs produced varying degrees of disintegration of the slope and a smell of hydrogen sulphide. The effect was not sufficiently clear cut, however, for this test to be substituted for the black rot production.

Growth at +1°. The following strains showed good growth on agar in 7 days at +1°: nos. 17, 95, 97–138, 140, 151, 152, 155, 157, 159, 160 and 170. Strains 16, 18, 96, 139, 141, 147, 158, 161–165, 172 and 173 showed slight growth in 7 days; strains 142, 144, 174 and 175 showed no growth at 7 days but slight growth at 14 days; and strains 143, 145, 146, 148–150, 153, 154, 156, 166–169 and 171 showed no growth in 14 days.

Pathogenicity for goldfish and mice. Single goldfish were inoculated with 0·2 ml of a suspension of bacteria centrifuged from a broth culture and resuspended in the same volume of saline. Most of the fish died within 48 hr of injection (Table 3). Only two of the ten strains in group III killed goldfish: no. 155 and the aberrant, gas-producing no. 113. The apparent lack of pathogenicity of strains 140 and 171 (*Aer. punctata*) is noteworthy, since this species is pathogenic for carp to which group the goldfish belongs. However, strain 140 has been shown to be atypical also in respect of its V-P reaction and inability to reduce nitrate, and Schäperclaus (pers. comm.) has said that strain 171 is less pathogenic than strain 170.

The following cold-tolerant strains were injected into pairs of mice: nos. 17, 95, 98, 112–119, 140, 151 and 152. All the mice except those injected with strains 113 and 140 died within 24 hr. One of the mice injected with strain 140 died within 48 hr but the other and those injected with strain 113 survived. Although only one goldfish was used for each strain and large doses were given, it is interesting to note that strain 113 killed the goldfish but not the mice, and that one of the mice injected with strain 140 died whereas the goldfish survived.

Table 4. *The reactions of lactose-fermenting* Aeromonas *strains at* 37° *and* 30° *in two broth media and their colony colours on two agar media*

Strain	Reactions* in broth media				Colony colour on agar media			
	MacConkey's		Brilliant green-bile		MacConkey's no. 3		Desoxycholate-citrate	
	37°, 24 hr	30°, 48 hr	37°, 24 hr	30°, 48 hr	37°, 24 hr	30°, 48 hr	37°, 24 hr	30°, 48 hr
95	g A	g A G	g	g G	Red centred	Red centred	Bright red	Bright red
98	g	g A tr G	g	g	Pink	Pink	Pink	Pink
114	g	g	g	g	Pink	Pale pink	Pink	Pale pink
116	g	g	g	g	Pink	Pale pink	Pink	Pale pink
144	g	g	g	g	Faintly pink	Faintly pink	Pale pink	Pale pink
145	g	g	g	g	Pink	Pale pink	Pink	Pale pink
149	g	g	g	g	Pink	Pale pink	Pale pink	Pale pink
150	g	g	g	g	Colourless	Pink	Colourless	Pale pink
155	g	g	g	g	Colourless	Pink	Red	Red
157	g	g	g	g	Colourless	Pink	Red	Red
159	g	g	g	g	Colourless	Red centred	Pink	Red
172	A	A	g	g	Red	Red	Red	Red

* g, growth; A, acid; G, gas; tr, trace.

Growth and reaction on media diagnostic for coli-aerogenes bacteria. Many of these media depend on the ability of these organisms to ferment lactose. Only the lactose-fermenting strains of *Aeromonas*, therefore, were tested in MacConkey's broth and brilliant green-bile broth, and on MacConkey's agar no. 3 and desoxycholate-citrate agar. Incubation was at 37° for 24 hr and 30° for 48 hr. The results are shown in Table 4. In all cases where growth took place it was better at 30° than at 37°.

All the strains were tested on the CNBLM medium of Mossel (1957) which was devised for the detection of most Enterobacteriaceae, whether fermenting lactose or not, following the suggestion by Henriksen (1955) that the lactose in diagnostic media be replaced by mannitol. All the strains tested were positive at 37°, except 141 and 161–164, which failed to grow, and at 30° except 162 and 163, which failed to grow at this temperature also. In all cases where growth occurred it was better at 30° than at 37°, and in many cases at 30° the positive reaction became reversed in 48 hr except in the isolated colonies.

(c) *Discussion*

The discussion of the results obtained with the various strains is arranged under three heads. The first deals with their systematic relationships one with another. The second deals with their pathogenicity to cold- and warm-blooded animals. The third is concerned with their ability to grow on refrigerated foods and the likelihood of their being confused with members of the Enterobacteriaceae in quantitative examinations for these organisms, especially as regards faecal contamination.

Systematic relationships

The strains will be treated in two series, viz. the Voges-Proskauer positive strains which may be taken to conform to the original criterion of Kluyver & van Niel (1936), and the V-P negative strains which do not conform in this respect and which include the nonmotile *Aer. salmonicida* and the strains of *Kluyvera* and *Photobacterium.*

Voges-Proskauer positive strains

(i) As regards those of group II, it is clear that because of their peritrichous or doubtful flagellation and distinctive biochemical reactions (Table 3)—in particular their failure in the oxidase and arginine tests, and their inability to hydrolyze casein, to ferment dextrin, starch and glycogen and to produce indole—strains 97 (*Aer. liquefaciens* L 418), 108 (Cook, 1958), 158 and 160 cannot be classified as *Aeromonas* and therefore fall outside the scope of this discussion. Hence they will not be discussed further, though it may be remarked that the description of *Aer. liquefaciens* in Bergey's *Manual* (7th ed.) will need to be revised since it is based partly on results obtained by Miles & Miles (1951) with strain 97 (L 418); for example, production of acid from raffinose and inositol and absence of haemolysis.

(ii) The V-P positive strains of group I, in addition to being polarly flagellated and able to produce acetylmethylcarbinol, have the following positive and negative characters in common. (a) Positive reactions: fermentation of glucose (with gas production), maltose, trehalose, fructose, galactose, mannose, mannitol, glycerol, dextrin, starch and glycogen (not no. 16); production of H_2S in ferrous chloride-gelatin; reduction of nitrate; production of indole (not no. 138); catalase; anaerobic production of NH_3 from arginine; oxidase; hydrolysis of gelatin; hydrolysis of casein; growth in the presence of KCN; sensitivity to terramycin (not no. 112); and production of black rot in hens' eggs. (b) Negative reactions: fermentation of raffinose, xylose, adonitol, dulcitol, inositol and inulin; urease; utilization of malonate; and sensitivity to penicillin and the vibriostat.

The following reactions are variable: fermention of cellobiose, lactose, sucrose, rhamnose, arabinose, sorbitol and salicin; methyl red test; utilization of citrate; hydrolysis of aesculin; and production of a filtrable haemolysin. The frequency with which cellobiose, sucrose and arabinose were fermented, citrate was utilized and a filtrable haemolysin was produced can be seen from Table 3.

These strains contain representatives of the three motile species listed in Bergey's *Manual*, 7th ed. There seems to be no justification on biochemical grounds for segregating these strains into different species, particularly in view of the frequency with which aberrant strains are encountered in the group which they most closely resemble biochemically, i.e. the Enterobacteriaceae (Report, 1958). Using the key to the species in Bergey's *Manual* (7th ed.) it is impossible to give a species name to any freshly isolated V-P positive strain. The differences between the results obtained here and those of Wunder & Dombrowski (1953) have been ignored, reliance being placed on the opinion of Schäperclaus (1958) that their results were due to the use of impure cultures.

Voges-Proskauer negative strains

Unlike the V-P positive strains, the V-P negative strains do not form a homogeneous group (Table 3, groups III, IV and V). Except for those of *Kluyvera* (group IV), none of the V-P negative strains gave positive results in tests which were consistently negative with the V-P positive strains, i.e. fermentation of raffinose, xylose, adonitol, dulcitol, inositol and inulin; urease; utilization of malonate; sensitivity to penicillin and the vibriostat were all negative.

(i) The V-P negative strains falling in group III mostly correspond biochemically to the *Ps. formicans* of Crawford (1954): they also closely resemble the V-P positive strains, though with the exception of strains 113 and 140 they do not produce gas. They differ, however, both from the V-P positive strains, and among themselves, in their action on casein, gelatin, serum and blood and in the production of H_2S in ferrous chloride-gelatin. Their lytic powers are in general less marked than those of the V-P positive strains. As suggested in Part 3, representative V-P negative strains were tested for the production of 2:3-butanediol. None was detected, thus confirming the interpretation of V-P negative reactions as the inability of the bacteria to carry out a 2:3-butanediol fermentation.

The anomalous strains in this group are nos. 113, 140 (*Aer. punctata*), and 174 and 175 (*Photobacterium*). Strain 113 fits well into the group apart from its ability to produce gas, and it may therefore be provisionally regarded as an aberrant gas-producing strain belonging to this group. Strain 140 also produces gas but, although it is V-P negative, it was obtained as *Aer. punctata* (CCEB 386). Since V-P negative strains of characteristically positive genera of the Enterobacteriaceae are not uncommon (Report, 1958), and since Schäperclaus (1958) recognized the existence of V-P negative strains of *punctata*, there seems to be little justification either for questioning the original designation or for segregating this strain from the main body of V-P positive strains which includes two other representatives of this species (strains 170 and 171). The problems raised by strains 113 and 140, and the somewhat arbitrary solution adopted here, indicate that, as in the Enterobacteriaceae, the taxonomic groups are not unequivocally delimited and that strains are likely to be found whose classification at the species level may be a matter of some difficulty. Strains 174 and 175 (*Photobacterium*) raise further difficulties. Leaving aside the questions of their moderate halophilism and luminescence they fit well into group III except for their failure in the arginine test. Since the taxonomic significance of these characters in species differentiation is not understood, and since only two strains are involved, it seems reasonable to consider them provisionally as belonging to group III.

(ii) The V-P negative strains of group IV yielded results which show clearly (especially those given in Table 3) that *Kluyvera* is totally unrelated both to the typically V-P positive *Aeromonas* and to the V-P negative *Ps. formicans* which was considered by Asai *et al.* (1957) to belong to it. Stained preparations of *Kluyvera* showing the flagella have not been easy to obtain because of the presence of glutinous extracellular material, but those which have been obtained have indicated that the strains are in fact peritrichous, bearing one or more flagella arising from all parts of the cell. Confirmation was obtained by electron microscopy (Plate 1).

(iii) As shown by the V-P negative group V strains, the systematic position of *Aer. salmonicida* is somewhat difficult, since in its lack of motility and its inability to produce acetylmethylcarbinol this species fails to meet the two criteria of the genus stipulated by Kluyver & van Niel (1936). It differs from the V-P positive strains in its morphology, in being nonmotile, V-P negative and indole negative, in producing a characteristic diffusible brown pigment on agar, in failing to grow in the presence of KCN, in not producing black rot in eggs and in being less able to attack cellobiose, sucrose, trehalose, mannose and arabinose. Nevertheless its strains resemble those in groups I and III sufficiently closely for them all to be included in the same genus.

The systematic placing of *Aer. salmonicida* and the *formicans* strains raises two alternative issues. Apart from the question of flagellation, if *salmonicida* is to be retained in the genus *Aeromonas* and *formicans* placed in it, which seems appropriate (Pivnick & Sabina, 1957), the definition of the genus must be revised so that the production of gas and 2:3-butanediol are no longer essential characters. Alternatively, if the definition of the genus is to stand, then *salmonicida* must be placed in a genus which is characteristically fermentative, V-P negative and nonmotile, and *formicans* in a genus which is characteristically fermentative, anaerogenic, V-P negative and cephalotrichous.

Pathogenicity

The genus *Aeromonas* includes types known to be pathogenic for a wide range of cold- and warm-blooded animals, from fish to man. Several of the named species were, in fact, isolated because they were pathogenic. It was therefore desirable to know which of the strains not isolated from diseased animals were pathogenic and for this reason inoculation experiments with goldfish were carried out. In addition to this, as several of the strains have been shown to be able to grow at +1° and therefore to be potential contaminants of cold-stored food, it was advantageous to know whether or not these cold-tolerant strains were pathogenic to warm-blooded animals.

Bearing in mind that large doses were given and that only one goldfish was used for each strain, the ability to kill these fish seems to be strong in group I, weak in group III and intermediate in group V. However, only comparatively few strains of the two latter groups were tested and the more critical investigation of further strains would be needed before pathogenicity or lack of it could be shown to be linked to either group.

Possibly more interesting is the question of the pathogenicity of these strains for warm-blooded animals. Fourteen cold-tolerant strains were each injected into a pair of mice and in all but two cases both mice died within 24 hr: the two less pathogenic strains (113 and 140) were the only two of those tested which do not produce a filtrable haemolysin. There exists therefore the possibility that strains of *Aeromonas*, some of which are able to grow well at low temperatures and might be expected to grow on chilled meats, from which in fact one was isolated (strain 95), are pathogenic to warm-blooded animals and perhaps to man. None of the strains known to have been isolated from human sources (147–151) was able to grow at +1°.

The experiments with mice were clearly only of a preliminary nature. It is hoped to make a critical investigation of the pathogenicity towards mice of some of the *Aeromonas* strains the subject of a later publication.

Relation to food hygiene

It was mentioned, in connection with the pathogenicity of these bacteria, that some were able to grow well at low temperatures and that one strain was isolated from chilled eviscerated chicken stored at +3°. Others were isolated from artesian well water being used in a poultry processing plant and the evidence from the literature suggests that one of the main habitats of these organisms is water. It is therefore probable that they would occur on chilled foods and equally likely that, if isolated, most of them would be dismissed as paracolons, particularly if incubation were at 30° rather than at 37°, a practice which is becoming increasingly common. Further, all the *Aeromonas* strains gave a positive result on the CNBLM medium recommended by Mossel (1957) for the detection of Enterobacteriaceae, whether able to ferment lactose or not; hence *Aeromonas*, which is clearly not related to this family, may have been present but overlooked when this medium has been used (e.g. Krol & Moerman, 1959–60).

If some types of *Aeromonas* organisms are shown to be pathogenic, the problem of their differentiation from nonpathogenic coli-aerogenes bacteria becomes a matter of some importance. The most likely way for this to be achieved seems at present to lie in the use of a combination of the oxidase test and the blood-milk agar mentioned at the beginning of the section on Methods.

The range of aberrant and atypical strains recognized in the coli-aerogenes group of the Enterobacteriaceae is so great (Thomas, Hobson & Druce, 1959) that, if the flagellation is not determined and the recommendations in Report (1956) are followed, it is possible to classify in the coli-aerogenes group the *Aeromonas* strains described here. For example, using the terminology adopted by Thomas *et al.* (1959) the Voges-Proskauer positive strains would be indole positive, 37° negative, citrate positive or negative *Klebsiella cloacae* or *K. cloacae* paracolon; strains 141 and 161–164 would be 37° negative, gelatin positive *Escherichia coli* II paracolon; strains 113 and 140 would be 37° negative, gelatin positive *E. coli* III paracolon; strains 155, 157 and 159 would be 37° negative, gelatin positive, anaerogenic *E. coli* III; strain 18 would be 37° negative, gelatin positive, anaerogenic *Citrobacter freundii* II paracolon; and strain 156 would be 37° negative, anaerogenic *Cit. freundii* II paracolon. The possibility exists therefore of strains of *Aeromonas* being classified as coli-aerogenes bacteria, and it is suggested that the oxidase test of Kovacs (1956) should be used in addition to the tests recommended in Report (1956), especially when aberrant or atypical strains are encountered.

5. CONCLUSIONS

It is clear from such evidence as positive oxidase and arginine reactions and similar patterns of fermentation that groups I, III and V (Table 3) constitute a biochemically, though not morphologically, homogeneous group of organisms for which the present system of classification (Bergey's *Manual*, 7th ed.) is inadequate. The present writer agrees with Spencer (1955) and Pivnick & Sabina (1957) that these bacteria should all be included (at least for the time being) in the genus *Aeromonas*. This would involve a revision of the description of the genus so as to remove the references to gas production, 2:3-butylene glycol production (cf. Miles & Miles, 1951), the methyl red test and the fermentation of lactose. It is suggested that the positive oxidase reaction, the ability to ferment dextrin, starch and glycogen and the inability to ferment raffinose, xylose, adonitol, dulcitol and inositol might be included.

If a revision of the characters of the genus *Aeromonas* is needed, a revision of its subdivision into species is even more necessary. The groups into which the strains described have been arranged may be taken as a basis for division into three species, as follows.

(i). Group I. This consists of polarly flagellated, Voges-Proskauer positive strains which cannot be differentiated biochemically though there may be differences of pathogenicity. These strains conform to the original criteria of Kluyver & van Niel (1936). It is suggested that these strains constitute a single species of *Aeromonas*, for which the specific epithet *liquefaciens* would appear to have priority, and which would include organisms previously known as *Aer. hydrophila* and *Aer. punctata*.

(ii). Group III. This consists of polarly flagellated, Voges-Proskauer negative, anaerogenic strains whose lytic powers are less marked than those of the other two groups. It is suggested that the name *Aer. formicans* Pivnick & Sabina be used for these strains.

If *Photobacterium harveyi* and *Ph. sepia* are to be accepted as *Aeromonas* spp. (Spencer, 1955) then, by virtue of their motility, their production of indole, their negative V-P reaction and their inability to produce gas from carbohydrates, they must be included in this species, at least until the taxonomic significance of their halophilism, luminescence and failure in the arginine test is decided.

(iii). Group V. This consists of nonmotile, Voges-Proskauer negative, gas-producing strains corresponding to *Aer. salmonicida*. It is suggested that this name should be retained. Although there is a precedent for including this species in the genus *Aeromonas* (Bergey's *Manual*, 7th ed.) it should be realized that, in their lack of polar flagellation and their inability to produce 2:3-butanediol its members fail to conform in two of the main characters which led Kluyver & van Niel (1936) to create the genus.

No evidence has been forthcoming from this investigation in support of Stevenson's suggestion (Stevenson, 1959) that *Aeromonas* can be regarded as nonpigmented *Serratia*. A study of Stevenson's own strains has shown that they differ from *Aeromonas* in failing to ferment dextrin, starch and glycogen, to produce ammonia anaerobically from arginine, to give a positive reaction in Kovacs' oxidase test, and to produce indole. Moreover they are peritrichous (W. Hodgkiss, pers. comm.). These strains cannot therefore be confused with *Aeromonas*.

Kluyvera, in addition to being peritrichous and giving a negative reaction in Kovacs' oxidase test, is clearly quite distinct biochemically from *Aeromonas*. The strains examined would seem to belong to the Enterobacteriaceae, but are difficult to classify within that group because of their utilization of malonate. However, on the basis of the IMViC reactions and gelatin liquefaction (Report, 1956), *Kluyvera citrophila* would be *Citrobacter freundii* II and *K. noncitrophila* would be *Escherichia coli* II.

The relationship of the genus *Aeromonas* to other groups of bacteria raises interesting questions. On the one hand it is related to the Pseudomonadaceae (in which it is placed at present) by virtue of its polar flagellation, positive oxidase reaction and strong proteolytic powers. On the other hand it resembles the Enterobacteriaceae in its strong fermentative ability. If, as has been suggested, the peritrichous bacteria evolved from monotrichous types, it may be that the *Aeromonas* group represents an intermediate stage in the evolution of the Enterobacteriaceae from the Pseudomonadaceae.

A suggested revision of the section on *Aeromonas* in Bergey's *Manual* (7th ed.) follows: two factors should be borne in mind concerning it. First, it comprises only such types as have already been described, additional descriptive material being derived only from those strains studied in the present paper. Second, the discovery that some pseudomonads may be oxidase negative suggests that oxidase negative aeromonads may also exist: only oxidase positive types are described in this paper, but no strain has been rejected solely because of a negative oxidase reaction. If oxidase negative types are found it may be necessary to create further species, as may also be required if types are found which are significantly different biochemically from those already described.

6. A SUGGESTED REVISION OF THE SECTION ON *AEROMONAS* IN BERGEY'S *MANUAL* (7TH ED.)

Genus IV Aeromonas *Kluyver and van Niel*, 1936 (*Zbl. Bakt.* (Abt. 2) **94**, 398)

A.e.ro.mo'nas. Gr. mas.n. *aer* air, gas; Gr. fem.n. *monas* unit, monad; M.L. fem.n. *Aeromonas* gas (-producing) monad.

Short (rarely more than 3 microns), rod shaped cells. Motile by means of polar flagella, usually monotrichous; or nonmotile. Gram-negative. Heterotrophic, oxidizing various organic compounds. Carbohydrates fermented, i.e. acid or acid and gas produced anaerobically. Dextrin and starch attacked; raffinose, xylose, adonitol, dulcitol and inositol not attacked. Give a positive reaction in Kovacs' oxidase test. The majority of strains thus far described are from water or are known to be pathogenic to marine and fresh-water animals such as fish and amphibians. Some strains have been isolated from human sources.

Physiologically these organisms closely resemble certain species found in the Enterobacteriaceae. The chief differences between late- or non-lactose-fermenting *Aeromonas* and late- or non-lactose-fermenting coli-aerogenes bacteria are found in the arrangement of their flagella, in the production of oxidase by the former, and in their pathogenicity.

The type species is *Aeromonas liquefaciens* (Beijerinck) Kluyver and van Niel.

Key to the species of the genus Aeromonas

I. Motile. Indole produced.

A. Acetylmethylcarbinol produced. Gas produced from carbohydrates.

1. *Aeromonas liquefaciens*

B. Acetylmethylcarbinol not produced. Gas not produced from carbohydrates.

2. *Aeromonas formicans*

II. Nonmotile. Indole not produced.

3. *Aeromonas salmonicida*

1. *Aeromonas liquefaciens* (Beijerinck, 1900) Kluyver & van Niel, 1936. (*Aerobacter liquefaciens* Beijerinck, *Zbl. Bakt.* (Abt. 2) **6**, 1900, 199; Kluyver & van Niel, *Zbl. Bakt.* (Abt. 2) **94**, 1936, 399. *Pseudomonas fermentans* von Wolzogen Kühr, *Zbl. Bakt.* (Abt. 2) **85**, 1932, 223. *Aeromonas hydrophila* Chester, *Manual Determ. Bact.* 1901, 235; Stanier, *J. Bact.* **46**, 1943, 213. *Proteus melanovogenes* Miles & Halnan, *J. Hyg., Camb.*, **37**, 1937, 79. *Bacillus ichthyosmius* Hammer, *Res. Bull. Ia agric. Exp. Sta.* no. 38, 1917. *Aeromonas punctata* Zimmerman, *Ber. naturw. Ges.* Chemnitz **1**, 1890, 38; Snieszko, Bergey's *Manual*, 7th ed., 1957, 190. *Vibrio jamaicensis* Caselitz, *Z. Tropenmed. u. Parasit.* **6**, 1955, 52.)

li.que.fa'ci.ens. L. v. *liquefacio* to liquefy; L. part. adj. *liquefaciens* liquefying.

Description taken from Beijerinck (*Zbl. Bakt.* (Abt. 2) **6**, 1900, 199), from Miles & Miles (*J. gen. Microbiol.* **5**, 1951, 299) and from Eddy (this paper).

Rods, 0·4 to 0·8 by 1·5 to 3·0 microns, with parallel sides and rounded ends. Filaments common in 'rough' colony forms. Motile by means of a single polar flagellum about 5 to 6 microns long. Gram-negative.

Gelatin stab: liquefaction rapid and commonly saccate, good growth.

Horse blood agar colonies; 2 to 3 mm in diameter, round, entire, raised, smooth, moist, opaque, semitranslucent, greyish-white, forming a dirty brown-yellow colouration after 3 to 5 days at room temperature; marked β-haemolysis. Filtrable haemolysin usually produced.

Broth: growth abundant, turbid, with a moderate, readily disintegrable sediment and delicate pellicle.

Loeffler's serum: growth abundant; serum cleared and partially digested.

Casein: digested.

Litmus milk: peptonized.

Potato: growth abundant, yellowish to reddish-brown.

Indole is produced.

Nitrites produced from nitrates.

Acetylmethylcarbinol produced.

Methyl red test variable.

Citric acid and salts of citric acid may be utilized as sole source of carbon.

Sodium malonate not utilized as sole source of carbon.

Ammonium sulphate, uric acid and asparagine may be utilized as sources of nitrogen.

Catalase produced.

Oxidase: positive by Kovacs' test (*Nature, Lond.*, **178**, 1956, 703).

Hydrogen sulphide produced in ferrous chloride-gelatin.

Urea not attacked.

Methylene blue reduced.

Starch hydrolyzed (Miles & Miles); starch not hydrolyzed (Beijerinck); starch fermented (Eddy).

Acid and gas from glucose, fructose, galactose, mannose, maltose, trehalose, glycerol, mannitol, dextrin, starch and glycogen. Fermentation of cellobiose, lactose, sucrose, arabinose, rhamnose, sorbitol and salicin variable. Raffinose, xylose, adonitol, dulcitol, inositol and inulin not attacked.

Aesculin hydrolysis variable.

Grows in the presence of $1\cdot1\times10^{-3}$ M potassium cyanide.

Aerobic, facultative.

Temperature relations: optimum about 30°; some strains grow well at +1°.

Produces a characteristic black rot in hens' eggs.

Pathogenic to mice, frogs, goldfish and other animals. May be pathogenic to man.

Source: found rarely in canal mud, generally in certain marshes and swamps.

Habitat relationships uncertain but probably primarily a water organism.

2. *Aeromonas formicans* (Crawford, 1954) Pivnick & Sabina, 1957. (*Pseudomonas formicans* Crawford, *J. Bact.* **68**, 1954, 734; Pivnick & Sabina, *J. Bact.* **73**, 1957, 247.)

for'mi.cans. L.n. *formica* ant; M.L. adj. *formicans* ant-like i.e. producing formic acid.

Description taken from Crawford (*J. Bact.* **68**, 1954, 734), from Pivnick & Sabina (*J. Bact.* **73**, 1957, 247) and from Eddy (this paper).

Rods, 1 to 2 by 4 to 5 microns. Motile by means of a single, polar flagellum. Gram-negative.

Gelatin stab: liquefaction usual.

Agar colonies: 2 to 3 mm in diameter, low, circular, convex, smooth, shiny and white with entire margins and a butyrous consistency.

Horse blood agar: haemolysis variable—no filtrable haemolysin produced.

Broth: diffuse growth, no pellicle.

Loeffler's serum: usually cleared and partially digested.

Casein: sometimes digested.
Indole: usually produced.
Nitrites produced from nitrates.
Acetylmethylcarbinol not produced.
Methyl red test positive.
Salts of citric acid may be utilized as sole source of carbon.
Sodium malonate not used as sole source of carbon.
Catalase produced.
Oxidase: positive by Kovacs' test.
Hydrogen sulphide production slight.
Urea not attacked.
Starch hydrolyzed.
Acid but no gas from glucose, fructose, galactose, maltose, sucrose, glycerol, mannitol, salicin, dextrin, starch and glycogen. Fermentation of mannose, cellobiose, lactose, trehalose and arabinose variable. Raffinose, rhamnose, xylose, adonitol, dulcitol, inositol, sorbitol and inulin not attacked.
Aesculin hydrolyzed.
Grows in the presence of $1{\cdot}1 \times 10^{-3}$ M potassium cyanide.
Aerobic, facultative.
Temperature relations: optimum about 30°; grows at 42°; some strains grow at +1°.
Some strains produce a black rot of hens' eggs.
Pathogenicity not demonstrated.
Source: sewage.
Habitat relationships uncertain but probably primarily a water organism.

3. *Aeromonas salmonicida* (Lehmann & Neumann, 1896) Griffin, 1954. (Bacillus der Forellenseuche, Emmerich & Weibel, *Arch. Hyg., Berl.*, 21, 1894, 1; *Bacterium salmonicida* Lehmann & Neumann, *Bakt. Diag.* 1 Aufl. 2, 1896, 240; see Mackie, Arkwright, Pryce-Tannatt, Mottram, Johnston & Menzies, Interim Report of the Furunculosis Committee. Edinburgh: H.M.S.O. 1930; Griffin, *Trans. Amer. Fish. Soc.* 83, (1953), 1954, 241.)

sal.mo.ni'ci.da. L.n. *salmo*, *salmonis* salmon; L.v. suffix *-cida* from L.v. *caedo* to cut, kill; M.L. fem.n. *salmonicida* salmon-killer.

Description taken from Griffin (*Trans. Amer. Fish Soc.* 82, (1952) 1953, 129) and from Eddy (this paper).

Rods, 1·0 by 1·7 to 2·0 microns, with rounded ends, occurring singly, in pairs or in chains. Nonmotile. Gram-negative.
Gelatin stab: crateriform to infundibuliform liquefaction in 1 to 3 days; complete liquefaction in 7 days. Growth filiform, beaded, best at top. Medium turns light brown near the surface of old cultures.
Agar colonies: circular, punctiform in 24 hr and 1 to 2 mm in diameter in 4 to 5 days, convex, entire, semitranslucent. Colonies and medium turn brown in old cultures.
Agar slant: growth abundant, butyrous, glistening, filiform, opaque to transparent, odourless, colourless. A soluble, brown, melanin-like pigment forms in 3 to 5

days. A bright salmon-pink colour develops when β-2-thienylalanine is present (Griffin, Snieszko & Friddle, *J. Bact.* **65**, 1953, 658).

Colonies developed on trypticase agar quickly turn a violet-black colour after the addition of 1 per cent aqueous *p*-phenylenediamine (Griffin, *Proc. Gen. Meet. Soc. Amer. Bact. Boston* 1952, 53; also see *Vet. Med.* **48**, 1953, 280).

Broth: moderate to strong clouding; no ring or pellicle; moderate flocculent sediment. Medium may clear in the upper layers and some growth may adhere to walls of test tubes of old cultures.

Litmus milk: peptonized.

Casein: digested.

Rabbit (and horse) blood agar: β-haemolysis in 2 days. Filtrable haemolysin produced.

Loeffler's serum: cleared and partially liquefied.

Indole not produced.

Nitrites produced from nitrates.

Acetylmethylcarbinol not produced.

Methyl red test variable.

Sodium citrate does not serve as a sole source of carbon.

Sodium malonate does not serve as a sole source of carbon.

Catalase produced.

Oxidase: positive by Kovacs' test.

Hydrogen sulphide not produced or only weakly so.

Urea not attacked.

Starch hydrolyzed.

Acid and gas from glucose, fructose, galactose, maltose, glycerol, mannitol, aesculin, salicin, dextrin, starch and glycogen. Fermentation of trehalose, mannose and arabinose weak and variable. Raffinose, cellobiose, lactose, sucrose, rhamnose, xylose, adonitol, dulcitol, inositol, sorbitol and inulin not attacked.

Aesculin hydrolyzed.

Does not grow in the presence of $1{\cdot}1 \times 10^{-3}$ M potassium cyanide.

Arginine and methionine are essential for growth; asparagine and leucine are highly stimulative while lysine is only moderately so (unpublished data, Griffin).

Aerobic, facultative.

Temperature relations: optimum variable, between 20° and 30°. Some strains able to grow slowly at +1°. Maximum 34·5°.

Pathogenic for most freshwater fish, particularly those belonging to *Salmonidae*.

Source: from dead fish, of the family *Salmonidae*, taken from a fish hatchery in Southern Germany.

Habitat: found in freshwater lakes, streams, rivers and fish ponds throughout Europe and also in the United States and Canada. Causes a furunculosis in infected fish; also occurs in apparently normal fish.

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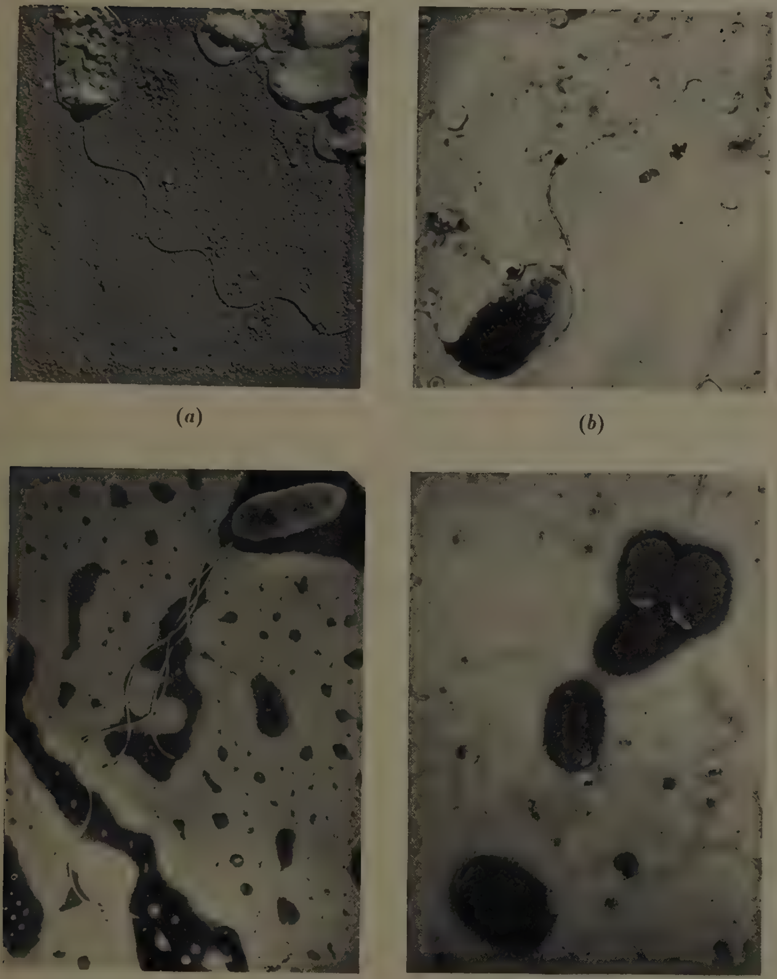

(*a*) (*b*)

(*c*) (*d*)

B. P. Eddy — Cephalotrichous Fermentative Gram-Negative Bacteria: The Genus *Aeromonas*. Plate 1

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Explanation of Plate

Electron micrographs of *Aeromonas* and *Kluyvera*

(a) Strain 113—*Aeromonas formicans*, showing typical single polar flagellum. Au–Pd shadowed, × 10,500.

(b) Strain 117—*Aeromonas liquefaciens*, showing insertion of single polar flagellum. Negative stain with phosphotungstic acid, × 15,000.

(c) Strain 167—*Kluyvera citrophila*, showing 3 subpolar flagella interpreted as being peritrichous (cf. Asai *et al.* 1957, Figs. 1C, 2B and 3A). Negative stain with phosphotungstic acid. × 7,500.

(d) Strain 168—*Kluyvera noncitrophila*, showing peritrichous flagellation. Negative stain with phosphotungstic acid, × 4,250.

Note Added in Proof

Since this paper was completed a further group of cephalotrichous, fermentative, oxidase positive, Gram-negative bacteria has begun to take shape. These are the C 27 strains (Ewing & Johnson, 1960 *loc. cit.*). They are V-P negative and anaerogenic but they differ markedly from *Aer. formicans*, especially in their ability to ferment inositol but not fructose, trehalose, mannitol, dextrin, starch or glycogen (Eddy, unpublished data), to produce ammonia anaerobically from lysine and ornithine, and in their inability to liquefy gelatin. *Aeromonas* seems to be the appropriate genus for them: their inclusion would necessitate removing mention of the fermentation of specific carbohydrates from the definition of the genus (p. 243), and the creation of a new species.

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THE DIFFERENTIATION OF BACTERIAL SPECIES BY PAPER CHROMATOGRAPHY. IX. THE GENUS *BACILLUS*: A PRELIMINARY INVESTIGATION

By D. J. JAYNE-WILLIAMS and G. C. CHEESEMAN

National Institute for Research in Dairying, University of Reading

SUMMARY: A preliminary survey of species of the genus *Bacillus* has shown that the comparative study of the bacterial amino pool, using paper partition chromatography techniques, shows promise as an aid to differentiation. But special factors, such as sporulation and other growth characteristics, make the use of these techniques more complicated than is the case with some other genera.

Seventy-five mesophilic strains were examined and in general the chromatogram patterns correlated well with the biochemical, morphological and cultural characters. Further, it appears that these chromatographic techniques could be applied with advantage in the study of spore germination, heat activation and sporulation, and of the influence of growth medium, pH, temperature and degree of aeration on the metabolism of micro-organisms. The taxonomy of the thermophilic sporeformers and of new species would be clarified by chromatographic studies.

The possibility of using the free amino pool of bacteria to differentiate species and strains has been investigated by Mattick, Cheeseman, Berridge & Bottazzi (1956), Berridge, Cheeseman, Mattick & Bottazzi (1957) and Cheeseman, Berridge, Mattick & Bottazzi (1957). Using paper partition chromatography they demonstrated that qualitative and quantitative differences in the amino pools of lactobacilli and streptococci could assist in differentiation. It was therefore considered worthwhile to determine whether the application of these techniques to the genus *Bacillus* would either confirm existing biochemical, morphological and cultural methods of differentiation or provide a new system of classification.

Most of the literature concerning the amino acid content of organisms of the genus *Bacillus* refers to total hydrolysates of cells, but in a few cases the free amino pools of a limited number of strains have been examined. However, no complete survey has yet been undertaken, nor has any attempt been made to utilize the information as a method of differentiation.

Stokes & Gunness (1946) studied the amino acid composition of hydrolyzed cells of *B. subtilis* (ATCC 6633) by microbiological assays and concluded from their results that this species occupied a position intermediate between *Escherichia coli* and *Staphylococcus aureus*.

Using specific amino acid decarboxylases, Freeland & Gale (1947) found lysine, glutamic acid, tyrosine, phenylalanine and histidine in cells of *B. brevis* and *B. subtilis*. The same method of assay was used by Taylor (1947) to determine the free amino acids of *B. subtilis* and *B. mesentericus* cells. Glutamic acid and lysine were found but arginine was not detected.

Evreinova (1948) hydrolyzed nucleoproteins extracted from the cells of a thermophilic sporeformer and compared the amino acid content with those of various mesophiles as determined by other workers; no differences were noted. Single dimensional paper chromatography was used by Proom & Woiwod (1949), who detected a wide range of ninhydrin positive substances in hydrolyzed cells of *B. subtilis*. Davis & Williams (1952), using paper chromatography, compared the amino acid content of hydrolyzed vegetative cells and spores of *B. globigii* with that of the medium in which they were grown. Tyrosine and methionine occurred in the spores only, phenylalanine, histidine and threonine in the spores and vegetative cells only, and hydroxyproline in the medium only. Numerous other amino acids were detected in all three materials.

Salton (1953) examined, by paper chromatography, the amino acid composition of the cell wall hydrolysates of various micro-organisms including *B. subtilis* (NCTC 3610), which was the only Gram-positive organism studied which contained diaminopimelic acid; however, all the Gram-negative organisms studied did so. Work & Dewey (1953) also showed that hydrolyzed cells of *B. subtilis* (and *B. pumilus* and *B. brevis* as well) contained this compound.

In seeking an explanation for thermophily, Bilimoria & Bhat (1955) determined the amino acid content of hydrolyzed cells of facultative thermophiles grown at 37° and 55°. No obvious differences were noted, but the suggestion was made that an examination of the free amino acids might show differences.

The free amino acid contents of *B. cereus* and *B. subtilis* cells were shown by Yoshida *et al.* (1957) to be similar in respect of lysine, aspartic acid, glutamic acid, the leucines and valine, but only *B. cereus* contained tyrosine and phenylalanine.

Reusser, Spencer & Sallans (1957) determined the essential amino acids present in various micro-organisms, including *B. subtilis* and *B. polymyxa*, by chromatographic analysis of acid hydrolysates (except for tryptophane which was assayed colorimetrically). Significant differences in the amino acid content of the two sporeformers were observed.

Biological assays of acid hydrolysates for lysine and methionine, and colorimetric determinations of tryptophane, were used by Anderson *et al.* (1958) to assess the content of these amino acids in various micro-organisms including sporeformers. Of the species of *Bacillus* examined, the two strains of *B. megaterium* had relatively higher contents of lysine and methionine.

Kandler, Hund & Zehender (1958) and Kandler & Hund (1959) studied the amino acid contents of bacterial cell membranes by chromatographic analyses of hydrolyzed material; a large selection of sporeformers was examined.

The utilization of amino acids by *B. subtilis* during growth and antibiotic formation was examined by Majumdar & Bose (1958), who showed that hydrolyzed cells contained a large variety of these substances.

Nakada, Matsushiro & Miwatani (1956) studied the incorporation of amino acids into the cells during the development of *B. cereus* and detected high concentrations in cells about to sporulate.

Vinter (1959) demonstrated that the spores of *B. cereus* and *B. megaterium* contained c. 5 times more cyst(e)ine (determined by chemical methods) than did the vegetative cells.

In this paper the results obtained from a limited survey of the genus *Bacillus* are described and some of the specific problems involved when the techniques of Mattick *et al.* (1956) are applied to the genus are discussed. To test the value of the techniques for the examination of this group of micro-organisms, preliminary experiments were carried out on a collection of organisms cultivated in a liquid medium. Despite the disadvantages which became apparent when a liquid medium was used, the results showed that the techniques were sufficiently promising to justify further experiments. These were carried out on cells harvested from agar media.

METHODS

Cultures. A collection of mesophilic species of the genus *Bacillus* was made from various sources. Concurrently with the chromatographic studies, the cultures were examined by a limited number of tests based on those described by Knight & Proom (1950) and Smith, Gordon & Clark (1952). The tests used and the typical biochemical, morphological and cultural (BMC) characters of each species are given in Table 1. Differentiation according to morphology was based on the following characteristics: group 1, spores oval and apparently thinwalled, causing little or no swelling of the sporangia; group 2, spores oval and apparently thickwalled, causing swelling of the sporangia; group 3, spores round and apparently thickwalled, causing swelling of the sporangia. Table 2 gives the sources and atypical characters of the cultures.

Preparation of material for chromatography. Cultures were grown in BS broth or BS agar (broth+2·5% of agar). The broth had the following composition: Bacto-tryptone, 3 g; peptone (Oxoid), 6 g; Yeastrel, 3 g; Lab-Lemco, 1·5 g; solution containing 0·001% (w/v) Mn^{++}, 1 ml; distilled water, 1 l.; pH 7·0.

The chromatographic techniques used were those described by Mattick *et al.* (1956).

Identities of spots. The chromatogram spots were identified when possible by their colour and position. The probable identities of all the spots encountered in the extracts of vegetative cells are shown in Fig. 1. For convenience each spot was identified by a number.

Recording of chromatograms. The method used was that described by Cheeseman & Silva (1959), the size and intensity of each spot being recorded by a single number in the scale 0–7.

Technique in experiments with broth grown cells. Quantities of 100 ml of BS broth in flasks containing glass beads were inoculated with *c.* 1% of active BS broth cultures of the test organism (24–48 hr at 37°). The flasks were placed on a reciprocating shaker in an incubator room at *c.* 33°. The cultures were examined periodically and, when growth was fairly well advanced, aliquots were removed aseptically for optical density (O.D.) determinations.

A preliminary experiment with one culture (*B. subtilis* 19) showed that cells harvested in the period between the beginning of the stationary phase of growth and the onset of sporulation showed little variation in extractable material. This is in agreement with the results of Berridge *et al.* (1957) who found the optimal stage for the harvesting of lactobacilli to be the beginning of the stationary phase. Accordingly cells were always harvested when the O.D. readings indicated that the stationary phase had been reached or when microscopic examinations of smears stained with dilute fuchsin indicated that sporulation was beginning. Some cultures were slow to grow or failed to produce spores; these were harvested after 40 hr incubation.

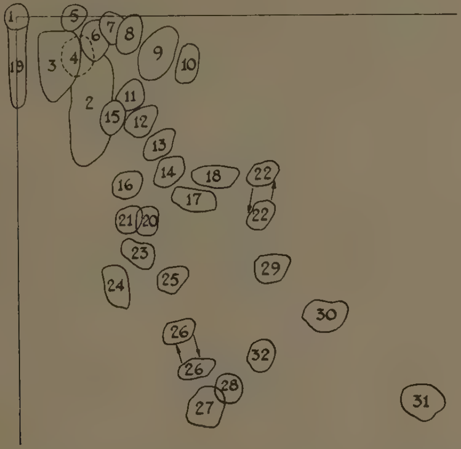

Fig. 1. The positions and identifications of chromatogram spots.

1. Origin
2. Lysine region
3. Unknown
4. Diaminopimelic acid
5. Possibly cysteic acid
6. Unknown
7. Unknown
8. Aspartic acid
9. Glutamic acid
10. Unknown
11. Serine
12. Glycine
13. Threonine
14. Alanine
15. Asparagine
16. Glutamine
17. β-Alanine
18. Unknown
19. Peptide streak
20. Hydroxyproline
21. Unknown
22. Tyrosine
23. Unknown
24. Probably arginine
25. Unknown
26. Unknown
27. Proline
28. Unknown
29. Aminobutyric acids
30. Valine-methionine
31. Leucines
32. Unknown

In all the broth experiments extraction of the washed cells was carried out with 10% acetic acid for 2 hr at room temperature (RT).

Technique in experiments with agar grown cells. During the experiments it became apparent that the cultivation of some of the test organisms in BS broth presented certain drawbacks. Many of the cultures (especially those of group 2 spore type) produced large amounts of slime which made centrifuging difficult and at times adversely affected the running of the chromatograms. Desalting the extract on ion exchange resin columns to remove the interfering material did not remedy the trouble. Because of these difficulties the main experiments were carried out with cultures grown on BS agar in Roux bottles. Each bottle was inoculated with *c.* 1 ml of an active broth culture (24–48 hr at 37°) and incubated at 37°. As successive O.D. measurements were not possible, the cultures were considered suitable for harvesting when microscopic examination of fuchsin-stained smears showed that sporulation was beginning. However, certain cultures sporulated rather slowly and these were harvested after 40 hr incubation.

For the cultivation of *B. pasteurii* the BS agar was fortified by the addition of filter-sterilized urea solution to give a final concentration of *c.* 2%. Urea solution was also added to the broth used for the preparation of the inocula.

Table 1. *The characters of* Bacillus *spp. as determined by the usual identification tests*

Species	Character of species								
	Spore type*	Production of acetoin from glucose	Starch hydrolysis	Gelatin hydrolysis§	Lecithinase† production	Anaerobic growth	Utilization of NH_4^+	CO_2 production from glucose	Additional tests‡
B. subtilis	1	+	+	+	—	—	+	—	
B. licheniformis	1	+	+	+	—	+	+	+	
B. pumilus	1	+	—	+	R	—	—	—	
B. firmus	1	—	+	+	—	—	—	—	citrate —
B. cereus	1	+	+	+	+	+	—	—	
B. megaterium	1	—	+	+	—	—	+	—	citrate +
B. lentus	1	—	—	—	R	—	—	—	
B. coagulans	1–2	+	+	—	—	+	—	—	PPAA +
B. pulvifaciens	2	—	—	+	—	+	—	—	
B. polymyxa	2	+	+	+	R	+	—	+	
B. macerans	2	—	+	+	R	+	—	+	
B. circulans	2	—	+	±	—	+	—	—	
B. alvei	2	+	+	+	R	+	—	—	
B. brevis	2	—	—	+	R	—	—	—	
B. laterosporus	2	—	—	+	R	+	—	—	
B. pantothenticus	2–3	—	+	+	R	+	—	—	
B. sphaericus	3	—	—	±	—	—	—	—	
B. pasteurii	3	—	—	±	—	+	—	—	

* See text. § ± Variable. † Egg yolk reaction; R, restricted. ‡ PPAA, proteose-peptone acid agar.

Harvesting was carried out by gently washing the growth from the surface of the agar with 10 ml of chilled, sterile water and glass beads. After thorough shaking by hand with beads in screwcap bottles the O.D.s of the suspensions were determined. The tendency of some cultures (notably *B. subtilis* and *B. licheniformis*) to grow in adherent sheets made harvesting difficult and sometimes resulted in suspensions which even after prolonged shaking were not uniform.

RESULTS

Experiments with cells harvested from broth

The chromatogram results obtained with the cultures harvested from broth generally showed interspecies differences large enough to justify further examination of the genus by this technique. As the patterns were in most instances similar to those obtained later with cultures harvested from agar, detailed reference to them will not be made. Interstrain variations in pattern were observed at times and were frequently indicative of wrongly named cultures, as shown by the BMC results (Table 2).

Table 2. *The numbers, sources and atypical biochemical, morphological and cultural (BMC) reactions of the organisms examined*

Species	Strain no.*†	Source‡	Atypical BMC reactions§
B. subtilis	3*, 4*, 18*, 19*	NCTC 3610, SM 786, NCIB 8057, NCIB 8159	
B. subtilis			
var. *niger*	266	CN 4008	
var. *aterrimus*	229	CN 2192	
B. licheniformis	68*, 82*, 87*, 94*	FI, NCTC 6346, FI, NCIB 7224	
B. pumilus	218*	CN 607	ABM+
	223*, 235*	CN 787, CN 807	
B. firmus	129*†	SM 564	ABM+, CIT+, (like *B. megaterium*)
	221*, 242*, 245	CN 2196, CN 1758, CN 2936	
B. cereus	108*	SM 585	ABM+
	98*, 112*	BC 60, FI	
B. cereus			
var. *albolactis*	103*	BC 484	
var. *thuringiensis*	227	CN 3625	LEC−, V-P weak+
var. *mycoides*	247	CN 1409	LEC−
var. *anthracis*	301	CN 1874	ST−
B. megaterium	116*, 125*, 128*, 226*	SM 697, SM 53, NIRD 128, CN 1759	
B. lentus	222*, 251*	CN 2750, CN 3321	
	246*	CN 2789	ST+, GEL+, AN+, (like *B. pantothenticus*)
B. coagulans	254, 259, 265, 295	CN 2919, CN 2835, CN 2832, NCTC 6301	
B. pulvifaciens	216*, 236*, 250*	CN 3621, CN 3623, CN 3622	
B. polymyxa	138*, 237, 258*	SM 1137, CN 1417, CN 2494	
	153*†	SM 1040	V-P−, LEC−, ABM+
	261*†	CN 3677	V-P−, ST−, AN−, CO_2−
	139	SM 920	LEC−, CO_2−
B. macerans	134*, 217*, 234*	SM 98, CN 2614, CN 1013	
	135*†	SM 367	ABM+, GEL−
	244	CN 2204	LEC−, ABM weak+
B. circulans	151*†	SM 368	CO_2+, V-P+, (like *B. polymxya*)
	158*†	NIRD 158	V-P weak+
	239*, 257*	CN 1609, CN 2201	
	290	NCTC 2610	Atypical spores
	292	NCTC 7578	V-P+, ST−
B. alvei	215*, 230*, 248*	CN 2771, CN 3186, CN 2198	ST−
B. brevis	228*, 253*, 260*, 288	CN 2904, CN 3010, CN 3005, NCTC 5098	

Table 2. (contd.). *The numbers, sources and atypical biochemical, morphological and cultural reactions (BMC) of the organisms examined*

Species	Strain no.*†	Source‡	Atypical BMC reactions§
B. laterosporus	219*, 267*, 268	CN 2197, NCTC 7579, NCIB 8213	
	272*	NCIB 8215	Atypical spores
B. pantothenticus	220*	CN 3019	LEC−
	233*	CN 3025	LEC−, ST−
	262*	CN 3043	LEC−, V-P+
B. sphaericus	156*, 240*, 256*	FI, CN 1652, CN 2514	
B. pasteurii	263, 264, 299	CN 2710, CN 2805, NCIB 8841	

* Cultures examined in preliminary experiments with broth grown cells; † cultures discarded as markedly atypical. ‡ Source: CN, the Wellcome Culture Collection; SM, Franklin *et al.* (1956); NCTC, National Collection of Type Cultures; NCIB, National Collection of Industrial Bacteria; BC, Stone & Rowlands (1952); FI, fresh isolate; NIRD, National Institute for Research in Dairying Collection. § Atypical reactions: ABM+, growth with NH_4^+ as sole source of nitrogen; CIT, citrate utilization; LEC, egg yolk reaction; ST, starch hydrolysis; GEL, gelatin hydrolysis; AN, anaerobic growth; V-P, production of acetoin; CO_2, formation of carbon dioxide from glucose.

The patterns of *B. firmus* 129 were very different from those of the other two strains examined. This was borne out by the BMC result which showed that the culture more closely resembled *B. megaterium* than *B. firmus*.

The BMC reactions of *B. lentus* 246 were similar to those of *B. pantothenticus*. These findings received support from the chromatogram results, which indicated the presence of spot 10. This was characteristic of group 3 organisms (*B. sphaericus* and *B. pasteurii*), *B. pantothenticus* and some strains of *B. coagulans*. It is of interest to note that the electron micrograph of spores of this strain of *B. lentus* published by Bradley & Franklin (1958) shows the spores to be very similar to those of *B. pantothenticus*, though these workers found the BMC reactions of this organism to be typical.

The chromatogram patterns of the other two strains of *B. lentus* were slightly dissimilar. Under the light microscope these two organisms differed quite markedly. Whereas the vegetative cells of strain 251 were long and the spores, which were oval and long, caused little or no swelling of the sporangia, the cells of strain 222 were much shorter and were swollen by short oval spores. Electron micrographs of the spores of these two organisms (Bradley & Franklin, 1958) also show differences. The spores of strain 251 appear quite smooth whereas those of strain 222 are rough.

The chromatogram patterns of the two atypical strains (153 and 261) of *B. polymyxa* differed from those of the typical strains and from each other. These two cultures were also examined by Bradley & Franklin (1958), who reported that the BMC reactions were typical. The electron micrographs, however, show that the spores differ from those of the other strains of *B. polymyxa* examined.

B. macerans 135 not only produced atypical BMC reactions but also chromatogram patterns which differed from those of the other strains examined.

Two strains of *B. circulans* (151 and 158) produced atypical BMC reactions and atypical chromatogram patterns. One of these (151) was also studied by Bradley &

Franklin (1958), who reported that the BMC reactions were typical. However, examination of the micrographs published by these workers shows that the spores are fairly deeply ribbed and thus resemble those of *B. polymyxa* more closely than those of *B. circulans*.

B. laterosporus 272, which failed to produce typical spores (but which was typical n all other respects) yielded a much stronger chromatogram than the other two cultures examined.

The strain (262) of *B. pantothenticus* which gave a positive V-P reaction also produced a chromatogram which differed in several respects from those of the other two cultures examined.

Experiments with cells harvested from agar

Preliminary experiments

Influence of degree of sporulation. Evidence in the literature suggests that nitrogen compounds of high molecular weight occur in spores and that sporulation is preceded by the formation of compounds of low molecular weight from vegetative cell protein (Hardwick & Foster, 1952). As sporulation proceeds there is a decrease in free amino acids, purines and pyrimidines (Foster & Perry, 1954). High levels of free amino acids in cells about to sporulate were observed by Nakada *et al.* (1956). Further, spores are generally impermeable to substances which can penetrate vegetative cells with ease. It thus seemed probable that the amount of extractable material in any culture would decrease as sporulation advanced. Experiments were carried out to confirm this.

First, clean mature spores of several species were treated with 10% acetic acid at room temperature (RT) for 2 hr; the extracts contained no ninhydrin positive materials. Second, replicate BS agar cultures of *B. licheniformis* 68 and *B. firmus* 221 were harvested after incubation at 37° for varying periods and the cells extracted at RT for 2 hr. The results (Table 3) showed that the amount of extractable material decreased as sporulation advanced.

Table 3. *The influence of degree of sporulation on the chromatogram intensities of the ninhydrin positive substances extracted from the cells of two* Bacillus *species*

Species, strain and degree of sporulation*		Intensities† of spots no.‡												
		1	2	3	7	8	9	12	14	16	17	26	27	29
B. licheniformis 68	a		1			1	4	1	1	1		1		
	c		1				4		1	1		1	1	
	d		1		1	1	4	1	1	2	1		1	
	e		1				2			1		1		
B. firmus 221	a	1	1	1		1	2			1		1		
	b	1	1	1		1	4		1	2		1		1
	c	1	2	2		1	4		2	2		2		
	d		1	1		2	2		1	2		1		
	e		1	1		1	1		1	1		1		

† Intensities, arbitrary scale 0–7. For clarity of presentation zeros have been omitted.

* Sporulation: a, none; b, very early; c, early; d, fair; e, marked.

‡ The identifications and positions of the spots are given in Fig. 1.

Influence of washing procedure. The rapid exchange of cell contents across the cell walls of Gram-negative organisms has been studied by Roberts *et al.* (1957). Experiments were therefore carried out to determine if this phenomenon were responsible for the weak chromatograms obtained with group 2 spore types, using *B. polymyxa* 237. At the same time tests were carried out on an organism (*B. pumilus* 223) which had previously yielded good chromatograms. The organisms were grown on BS agar at 37° and harvested in the manner already described. The supernatant solutions after the first centrifuging and first washing were spotted on to single dimensional chromatograms, as also were the final acetic acid extracts (2 hr at RT) and, as a control, the material extracted from a sterile bottle of BS agar. The agar was inoculated with 1 ml of sterile water, incubated and then washed with 10 ml of water. The entire acetic acid extracts, and 5, 10 and 15 drops of the other solutions, were applied to the chromatograms which were then irrigated with the butanol-acetic acid-water solvent.

The results are given in Plate 1. The washings of both organisms (c and f) were very weak; those of *B. polymyxa* (c) had the same basic pattern as the culture supernatants of both test organisms (b and e) and the washings of the sterile medium (a). The acetic acid extracts (d and g), however, had completely different patterns, and it is clear that no appreciable leakage of cell contents occurred during the washing procedures.

Influence of method of extraction. The use of a 2 hr extraction period was at times inconvenient, especially when cultures were harvested late in the day. Accordingly, the possibility of reducing the extraction time was investigated. Duplicate cell suspensions were prepared from 7 cultures harvested from BS agar. These were extracted for 2 hr at RT and for 30 min in a 37° water bath. The effect of these extraction methods on the chromatogram intensities and patterns is shown by the results given in Table 4. The two methods generally yielded similar results; the shorter procedure was therefore employed in all subsequent experiments.

Influence of the growth medium. The reason for using BS agar in the main experiments was that apart from the need for supplementation with urea for *B. pasteurii*, it supported good growth of all the cultures examined. But certain sporeforming bacteria are able to utilize NH_4^+ as the sole source of nitrogen (Knight & Proom, 1950) and limited experiments were therefore carried out on two organisms (*B. subtilis* 266 and *B. megaterium* 125) which could grow on the ammonia basal medium (ABM) of those authors. Sterile glucose solution was added to ABM agar and BS agar before sloping, to give a final concentration of *c.* 3%. The cultures were inoculated on to these two media, incubated and harvested when sporulation was just occurring. After washing, the cells were extracted with acetic acid and chromatograms prepared.

The chromatograms were all rather weak, possibly due to the presence of glucose (which might have resulted in unfavourable pH values). Those of *B. subtilis* 266 were identical for both media. However, *B. megaterium* 125 harvested from glucose-BS agar yielded a much weaker chromatogram than cells harvested from glucose-ABM agar.

Influence of aeration. As many species of *Bacillus* are facultative anaerobes a few experiments were done to determine the influence of aeration on the quantity and type of the extractable material. The medium used throughout was BS agar plus glucose. Three cultures (*B. licheniformis* 82 and 87 and *B. polymyxa* 258) were grown both aerobically and anaerobically.

Table 4. *A comparison of the chromatogram intensities of the ninhydrin positive substances extracted by two methods from the vegetative cells of various sporeformers*

Species, strain and method of extraction*		Intensities† of spots no.‡																				
		1	2	3	4	8	9	10	11	12	13	14	16	17	19	22	23	27	29	30	31	32
B. subtilis 3	a		3	1	(2)	1	6			1	1	3	2			1	1	1	1			
	b		2	?	3	1	6			1	1	3	2			1	1	3	2	1		
B. licheniformis 68	a		2	1		?	4			?	?	1	1	1				1				
	b		1	1	?	?	5			?	?	2	2	1				1				
B. megaterium 116	a	2	1	1	(2)	1	5			1	1	2	1	1		1	1	1	1	2	2	2
	b	1	3	3	(2)	1	5			?	1	3	1			2		2	2	1	?	1
B. macerans 134	a	4	3	3		3	2		1	1	1	1	1			2						
	b	3	3	3		2	2		?	1	?		1			1			?	?		
B. firmus 221	a	1	2	2		2	4					1	1									
	b	?	2	2		2	4					1	1									
B. laterosporus 267	a	5	1	2			2								1							
	b	5	1	1			1								1							
B. sphaericus 256	a	3	2	3		1	3	2				1	1									
	b	3	2	2		1	3	2			?	?										

* Extraction: a, for 2 hr at RT; b, for 30 min at 37°. † Intensities, arbitrary scale 0–7: ?, indicates trace only. For clarity of presentation zeros have been omitted. ‡ The identifications and positions of spots are given in Fig. 1; brackets indicate uncertainty of interpretation.

The chromatograms were all rather weak, possibly (as suggested above) due to the presence of glucose, but they were similar, which suggests that the amino pools are not affected by the atmospheric conditions used for cultivation of the organisms. To obviate the possible interference of glucose, the experiments should be repeated using a medium containing NO_3^- as the hydrogen acceptor in place of glucose.

Influence of temperature of growth. Two organisms (*B. licheniformis* 82 and 87), which were capable of growth at both 22° and 55°, were grown on BS agar at these temperatures and harvested when sporulation was commencing. Chromatograms were prepared from acetic acid extracts of the cells, and the preliminary results showed that the temperature of growth had a significant influence on the nature of the extractable material. With *B. licheniformis* 82, although the chromatogram for cells grown at 22° was slightly stronger than that for cells grown at 55° (glutamic acid scored 5 and 4, respectively), it showed in addition a strong β-alanine spot, fair amounts of proline, hydroxyproline, spot 21 and spot 23. The cells grown at 55° (unlike those at 22°) contained histidine. With *B. licheniformis* 87, the chromatogram for cells grown at 22° was again slightly stronger and showed (unlike the chromatogram for the cells grown at 55°) strong β-alanine and proline spots, fair amounts of spot 23, spot 24 and histidine; glutamine was twice as strong in the cells grown at 22°.

Similar experiments were carried out with 3 strains of *B. coagulans* grown at 37° and 55°, with the results shown in Table 5. It will be seen that although the lysine region (spot 2) had the same intensity for cells grown at both temperatures, cells grown at 55° generally contained more of spots 5, 26 and 10, and less aspartic acid (spot 8), glutamic acid (spot 9), serine (spot 11), glycine (spot 12) and threonine (spot 13) than cells grown at 37°.

Reproducibility of results. Duplicate experiments were carried out on a selection of 12 cultures to determine the reproducibility of the technique of harvesting from agar, using a 2 hr period of extraction at RT. The results showed that though variations between duplicates occurred, these were generally slight and might in some instances be attributed to differences in the degree of sporulation of the cultures and to lack of uniformity of some suspensions, whereby errors were occasioned in the O.D. readings.

Table 5. *A comparison of the chromatogram intensities of the ninhydrin positive substances extracted from vegetative cells of* B. coagulans *grown at 37° and 55° on agar*

Growth temp. (°C)	Strain	Intensities* of spots no.:†																								
		1	2	3	4	5	8	9	10	11	12	13	14	15	16	17	18	20	21	22	23	24	26	27	29	30
37°	254		2	3			2	7		?	2	2	3		4				?				1			
		2	1	1	2		2	5		2	4	2	2		3			?					1			
	295		4	1	(1)	2	4	5		3	4	4	2		3			2	?				2			1
			4	1		2	3	6		3	5	4	1		3	1		(2)	1				(3)			
	259		2	2	?		3	6	2	1	4	3	3	1	3						1	1	?		1	2
		1	3	2	2		3	5	3	2	4	3	3	?	3					2			1			1
	265	2	3	1	2		2	4	2	1	3	1	3		2	?	1						(2)	(1)		1
55°	254		3	2	(2)	2	1	6	(1)	3	4	3	3		3			4					2			
	295		3	2	4	2	1	3	1		1	1			2				1				3			
	259	1	2	1	4	(1)	2	4	2	1	1	1	2		1											

* Intensities, arbitrary scale 0–7: ?, trace only. For clarity of presentation zeros have been omitted.

† The identifications and positions of the spots are given in Fig. 1; brackets indicate uncertainty of interpretation.

Hydrolysis of vegetative cells and spores. The amino acids present in certain vegetative cells and spores were determined by analyzing complete acid hydrolysates. Washed vegetative cells (prepared as described above for acetic acid extracts) and spores (harvested from BS agar after incubation for 7 days at 37°) were suspended in 0·25 ml of conc. HCl. The suspensions were sealed in ampoules and heated for 24 hr at 110°. The hydrolysates were then desalted, either by passage through ion exchange resin columns or by distillation of the acid in an evacuated desiccator containing KOH pellets, and two-dimensional chromatograms prepared. The materials examined were: vegetative cells of *B. megaterium* 125, *B. brevis* 228 and *B. sphaericus* 156, and spores of *B. subtilis* 4, *B. coagulans* 254 and *B. sphaericus* 156. The chromatograms were virtually identical and showed the following amino acids, the relative intensities of which are indicated by the numbers in brackets: lysine (3), diaminopimelic acid (trace), aspartic acid (3), glutamic acid (3), glycine (3), serine (3), threonine (3), tyrosine (2), alanine (4), proline (3), valine-methionine (3), the leucines (3), arginine (1) and histidine (1). The chromatogram of the hydrolyzed vegetative cells of *B. megaterium* 125, however, contained a stronger spot (scored 2) of diaminopimelic acid.

Main experiments

The nature and intensity of the extractable ninhydrin positive materials in cells harvested from agar are shown in Tables 6–8. With the exception of markedly

Table 6. *The chromatogram intensities of the ninhydrin positive substances extracted from vegetative cells of group* 1 *sporeformers grown at 37° on agar*

Species and strain		Intensities* of spots no.†																												
		1	2	3	4	5	7	8	9	11	12	13	14	15	16	17	19	20	21	22	23	24	25	26	27	28	29	30	31	32
B. subtilis	3		3	1	(2)			1	6		1	1	3		2					1	1			?	1		1			
			2	1	(2)			1	6		?	1	2		1						1				1					
	4		3	1	(2)			1	5		1	1	2		2					1	1		(1)	(1)			?			
			3	2	?			?	7		1	1	2		3						?				2	1	1			
	18		2	1	2			1	5		1	1	2		2						1				2		?			
			2	1	(2)			1	4		1	1	2		2						1				1					
	19		3	1	2		(1)	1	5		1	1	2		2				(1)		1		(1)		?		?			
			4	1	(3)		2	1	7		1	1	3		3					(1)	1			?	1			2		
	226		3	1	?	1		1	4	?	?	1	3		1				1		1				2	1	1			
	229		1			(1)			4				1		1				?		2				2					
B. licheniformis	68		2	1				?	4		?	?	1		1	1					?				1					
			2	2			?	1	6		?	1	2		3	1					1				1		?	?		
	82		3	2	?			1	5	1	1	1	3		3	1			1	1	2	1			2		1	1		
		1	1	2				1	4	1	1	1	2		3	1					2			1	(1)					
	87	2	3	2	(2)			1	6	3	1	2	4	(1)	3	1			1		1	1			3		3	3	3	
		1	3	1	(2)			1	5	1	1	1	2		3	1				1	1	1			1	1		1	2	
	94	1	3	1	(1)			1	5	1	2	1	3	?	2				1		2				2		1			
			1	1				?	4	?		?	1		1						1				?					
B. pumilus	218		1	1				1	5	1			?		2			3												
			1	(1)	?			?	4				1		1			2												
	223		2	1	1			1	5				1		1				?		1			1	?		?	3		
			2	1	(2)			?	7				1		1						1				?					
	235			3	?	1	1	1	5	?		?	2		2		2	2			2			1	?	1		1		
			1	1	(1)		1	2	6	2		1	2		2			2	1					1	?			1		
B. firmus	221	1	2	2				2	4				1		1															
		1	1	?				1	4				?											1						
	242	1	4	3				2	5				3		1									2						
			3	1				1	6				1		2				?					1						
	245	2	1	1				1	3				1																	
		2	2	3				1	3			?	1			?		(2)												
B. cereus	98	2	1		2			1	4		1		3					?		1					1		1			
		2	1	2	?		1	1	5		?		2					?							1		?			
	103	1	1		2			1	5		?		2												?		?			
		1	1	2	(2)			1	4				2																	
	108	1	1		2			1	4				3												1		1			
		2	1	3	?		2	1	5	1		1	3																	
	112	2	1		2			1	4		1		3					?									?			
		2	1	2	(1)		1	1	5		?		2					(1)							?					
	227		2	1	2			1	5			1	3																	
	247		2	2	(1)			2	5		1	1	3																	
	301	2	1	2	?			1	5		1		2			1														
B. megaterium	116	2	1	1	(2)			1	5		1	1	2		1	1			1	1					1		1	2	2	2
			1	2				?	4		1	1	2		1	?									?		?	?		?
	125		3	3				1	5			1	2		2	1		?	1		?	1			3					
			3	2				1	6			1	2		2										3					
	128	1	1	1	(1)			1	4		1		2		1				1						2					1
			1	1				?	3		?		1		1			1							1					?
	226	1	1	1	(1)			1	4		?	?	2		2				1						1		1			1
			2	1	(1)			1	5				1		1				2						2					2
B. lentus	222		1	1				2	3	1	?	1	1																	
			2	2				3	5	2	1	2	2		1				1		2				1					
	251		2	1				(5)	3	2		2	1		2						1									
			2	?				4	2	1	1		1		(1)															

* Intensities, arbitrary scale 0–7: ?, trace only. For clarity of presentation zeros have been omitted.

† The identifications and positions of the spots are given in Fig. 1; brackets indicate uncertainty of interpretation.

atypical cultures, the strains examined were the same as those used in the experiments with broth grown organisms. Atypical cultures were replaced by other strains and, with *B. subtilis* and *B. cereus*, a number of varietal strains were studied (Table 2). The characteristic BMC reactions of the varietal strains were not confirmed. The culture originally received as *B. lentus* 246 has been included with *B. pantothenticus*.

Table 7. *The chromatogram intensities of the ninhydrin positive substances extracted from vegetative cells of group 2 sporeformers grown at 37° on agar*

Species and strain		Intensities* of spots no.†														
		1	2	3	8	9	11	12	13	14	16	19	20	23	26	27
B. pulvifaciens	216	4	1	2	2	4				3			3			3
		4	2	3	2	4			1	2			2			1
	236§	2	2	2	2	4			2	1			1			2
		3	2	2	1	4			1	2			2			2
	250	5	2	2	1	3				3			3			3
		3	2	2	2	5			1	2			2			2
B. polymyxa	138	2	2	2	1	2										
		3	2	2	1	1		1						1		
	139	3	1	1												
		2	2	1	1	1			?	?		1			?	
	237§	4	3	3	1	4				?						
		3	1	2	2	1				?						
	258§	3	1	2	?	1				1						
		3	1	2	2	1						1				1
B. macerans	134§	4	3	3	3	2	1	1	1	1	1			2		
		2	2	2	1	1			?	?				1		
	217	2	1	2		1										
		2	2	2	1	1									?	
	234	3	1	2	1	1										
	244§	3	2	3	2	1		1								
		2	1	1	1	1		?	?	?						
B. circulans	239	5	3	3	1	2		1	1	1	1					
		5	1	2	1	1		1				1				
	257	5	3	1		2		?								
		3	2	1		1		?				1				
	290§		2	1	3	4		(2)	(2)	(1)	?					
			1	2	3	3		2	(2)	?	(1)		2	1	1	
	292§		1	1	1	3		1							2	
			2	1	2	6	1	3			1					
B. alvei	215	6	1	1		1						1				
		5	2	3	1	2						1				
	230	5	1	1	?	1						1				
		4	2	2		1						1				
	248§	5	2	2	1	2	2	1	2	2	2			1		
		5	2	2	?	1										
B. brevis	228	5	1	1		2						1				
		5	1	1		?				?		1				
	253	4	2	3		2		(1)		1		1			(1)	
		6				?				1		3				
	260	5		1		1				(1)		1				
		4	2	1	?	2		1	?	1		1				
	288	4	1	2		1										
		2		1		1										
B. laterosporus	219	6	1	2	1	1	1	?				2				
		6	2	1		1						1				
	267	5	1	2		2						1				
		6	1	2		2						2				
	268	5	1	1	?	1	1					1				
		6	1	2	1	2	1	1		1	(2)	2				
	272	6	1	2	1	1	1	?				1				
		6	2	1		1						1				

* Intensities, arbitrary scale 0–7: ?, trace only. For clarity of presentation zeros have been omitted.

† The identifications and positions of the spots are given in Fig. 1; brackets indicate uncertainty of interpretation.

§ Certain spots (4, 7, 10, 17–21, 25, 30, 31) have occurred only in trace amounts in one duplicate of these strains and reference to them has been omitted from the Table.

Some of the salient interspecies differences shown in Tables 6–8 are as follows:—

Group 1 *spore type*. *B. licheniformis*, unlike *B. subtilis*, contained β-alanine (spot 17) and had a stronger proline spot (no. 27); *B. subtilis*, unlike *B. pumilus* and *B. firmus*, contained diaminopimelic acid (spot 4), glycine (spot 12) and proline (spot 27);

B. pumilus, in contrast to *B. firmus*, contained serine (spot 11) and had stronger alanine and glutamine spots (nos. 14 and 16). Of the two large-celled species, only *B. megaterium* contained glutamine (spot 16) and it also had stronger alanine and proline spots (nos. 14 and 27) than *B. cereus*.

Table 8. *The chromatogram intensities of the ninhydrin positive substances extracted from vegetative cells of group* 3 *sporeformers grown at* 37° *on agar*

Species and strain		Intensities* of spots no.†																			
		1	2	3	4	6	8	9	10	11	12	13	14	15	16	17	22	23	25	27	28
Bacillus pantothenticus	220		3		(2)		3	5	2				2		?						
			3	?			2	5	1	1			2		1	1					
	233		1				1	4	1												
			2				1	7	2				1		1			1			
	262		1				1	4	2				1		1						
			3	?			1	5	2				1		1						
	246		3	1			1	6	2			?	2		2						
			3	3	?		1	7	(2)	?	1	1	2	1	1		1		2		1
Bacillus sphaericus	156	3	3	3			2	3	2				1								
		2	2	3			1	1	1				?								
	240	3	2	3			1	3	2				1								
		2	2	3			1	2	1				?								
	256	3	2	3			1	3	2				1		1						
		2	1	2			?	1	1				?								
Bacillus pasteurii	263		2				2	4	3				1								
		2	2	1			1	2	1			1	1								
	264	1	2				2	4	1	1			2								
			2				1	3	1		1	1	2		(1)					(1)	
	299		3	3		2	3	4	3	1			2								
			1	2		1	1	3	2				1								

* Intensities, arbitrary scale 0–7: ?, trace only. For clarity of presentation zeros have been omitted.

† The identifications and positions of the spots are given in Fig. 1; brackets indicate uncertainty of interpretation.

Group 2 *spore type.* With the exception of two of the strains of *B. circulans* the patterns of the species of this group examined were characterized by strong origins (spot 1). However, one of these *B. circulans* strains was atypical (no. 292, Table 2). *B. pulvifaciens*, with its high contents of glutamic acid and alanine (spots 9 and 14) and exclusive contents of hydroxyproline (spot 20) and proline (spot 27), is distinct from the rest of this group whose patterns (excepting the strains of *B. circulans* mentioned above) were very similar.

Group 3 *spore type.* Spot 10 occurred consistently in all the species and strains examined. *B. pantothenticus*, unlike *B. sphaericus* and *B. pasteurii*, contained glutamine (spot 16) and had a stronger glutamic acid spot (no. 9), and spot 1 was absent. *B. sphaericus*, in comparison with *B. pasteurii*, had stronger spots 1 and 3 but less glutamic acid and alanine (spots 9 and 14).

The chromatogram patterns of representative strains of each species examined are shown in Plates 2–5.

DISCUSSION

The literature concerning the amino acid composition of aerobic sporeformers, an outline of which has been given above, concerns in the main analyses of hydrolyzed complete cells. However, it is clear from the results of Proom & Woiwod (1949) and Bilimoria & Bhat (1955) that there are no major differences between the amino acid contents of hydrolysates of widely differing species, and this view is supported by the present analyses of hydrolyzed vegetative cells and spores of different *Bacillus* species. But although different workers have shown hydrolyzed material to be generally similar in composition there are differences: e.g. Bilimoria & Bhat (1955) did not detect the leucines but, like Proom & Woiwod (1949), did find cystine, though Davis & Williams (1952) did not detect the latter. Such conflicts may have resulted from differences in technique or have been due to variations in concentration.

The literature contains only limited information about the free amino acid content of sporeformers. Yoshida *et al.* (1957) detected phenylalanine in *B. cereus*, but this was not found in any of the species examined here and the same workers recorded much higher concentrations of aspartic acid in *B. cereus* and *B. subtilis* than were found in these species in the experiments described here; further, they did not detect alanine, proline, threonine, glycine and diaminopimelic acid. The results given by Majumdar & Bose (1958) for the free amino compounds present in *B. subtilis* cells closely resemble those reported in the present paper; however, they found the leucines and valine-methionine in higher concentrations.

Chromatographic techniques, when applied to the examination of the free amino compounds of vegetative cells, yield an immense amount of information and it has been possible in the Results section of this paper to outline only the major differences between species and strains. Because of the already mentioned difficulties encountered when the group 2 spore types were grown in broth most of the experiments were done with cells harvested from agar, but this method also had a serious disadvantage, that uniform suspensions of certain cultures could not be obtained. This, it is believed, has resulted in such large inter-duplicate differences that small interstrain differences have not been observed. More uniform results were obtained with the group of sporeformers harvested from broth. Difficulties arising from the presence of slime, etc. in extracts, and from lack of uniformity of suspensions, are inherent in techniques based on O.D. readings. Similar problems would no doubt arise if the amount of cells to be extracted were standardized by colony counts, because of the differences in cell size which exist between species and between strains. One possible alternative would be to vary the amount of extract used for chromatography according to its content of ninhydrin positive material; colorimetric methods could be used to determine this in an aliquot of the extract. Another important factor which militates against close similarity between duplicates is the degree of sporulation of the cultures; this does not arise with most other genera of bacteria. The results given in Table 3 show that among the strains examined the amount of extractable ninhydrin positive substances decreased as the degree of sporulation increased, presumably because of the conversion of soluble substances into spore material of high molecular weight.

Despite the weak chromatograms obtained when the influence of aeration and composition of medium on the amino substances extractable from vegetative cells was being studied, sufficient evidence was obtained to suggest that these two factors had less effect than might have been expected. Significant differences occurred, however, between the chromatogram patterns of organisms grown at 22° and 55°. These may be a reflection of variations in nitrogen metabolism, of differences in the rate of turnover of intracellular materials, of unequal production of capsular material (which affects O.D. readings but does not contribute to chromatogram patterns) or of increased leakage (during growth or washing) of cell contents across the cell wall of organisms cultured at high temperatures. However, with *B. coagulans*, the differences in extractable material present in cells grown at 37° and 55° (Table 5) were not so consistent.

The results shown in Tables 5, 6, 7 and 8 demonstrate that certain nitrogenous compounds may occur exclusively, or in characteristically high levels, in certain species or groups of bacteria; e.g. the material responsible for spot 10 in group 3 organisms and in some strains of *B. coagulans*.

The difficulties encountered by others who have studied the classification of certain sporeformers are amplified by the present results. One of the 3 cultures received as *B. lentus* proved to resemble *B. pantothenticus*; from others quite distinct chromatogram patterns were obtained (Table 6 and Plate 3, a, b). The unusual ratio of aspartic acid (spot 8) to glutamic acid (spot 9) with strain 251 is particularly striking. Gibson (1935) considered that there was a relationship between *B. lentus* and *B. pasteurii*. Smith, Gordon & Clark (1946), however, on the basis of the examination of 1 strain (Gibson's no. 165), concluded that this species was somewhat related to *B. circulans*; nevertheless pending further study, they placed it near *B. firmus*. Knight & Proom (1950) studied 3 strains (including Gibson's no. 165) and found that they differed in several respects from *B. firmus*, but Smith, Gordon & Clark 1952), having re-examined Gibson's no. 165 and another (Gibson's no. 238), listed *B. lentus* as a separate species. Proom & Knight (1955) reported that the two strains used in the work described here differed in their nutritional requirements; when cultured in a complex amino acid medium, strain 222 unlike 251 required the addition of urea. It is clear from the chromatogram results (Table 7) that both the strains of *B. lentus* differ markedly from *B. pasteurii*, *B. firmus* and *B. circulans*. Examination of more strains is thus necessary before the position of *B. lentus* can be clarified.

Smith, Gordon & Clark (1952) studied a number of strains of *B. coagulans* and described the spores as "... subterminal to terminal, and the sporangia in some cases definitely swollen". It is not clear from this, however, whether spores of group 2 type were formed exclusively by certain strains or were formed together with group 1 type spores in individual cultures (*cf.* the production of both oval and round spores in individual cultures of *B. pantothenticus*). Knight & Proom (1950) placed all the strains they examined in group 1, but Franklin & Bradley (1958) found the strains they examined consistently to form either group 1 or group 2 type spores, but not both. On the basis of spore morphology it thus seems possible that there are two distinct types of *B. coagulans*; but the similarity of the cultural and biochemical reactions of group 1 and group 2 spore type strains of this species does not support this. The 4 strains of *B. coagulans* studied had similar biochemical and cultural characters but differed in spore morphology. Strains 259 and 265 formed thickwalled spores which caused slight swelling of the sporangia and were classed as group 2, strain 295 formed spores classed as group 1, but no definite conclusion could be reached about the spores of strain 254. Bradley & Franklin (1958) described the spores of strains 265 and 259 as group 2 and those of strain 254 as group 1. The chromatogram patterns (Table 5) of cultures grown at 37° were generally similar but interstrain and interduplicate differences were sometimes marked. One striking feature of the results is that spot 10 (found in the chromatograms of group 3 sporeformers) occurred in both the group 2 sporeforming strains, although traces of this material appeared in the chromatograms of group 1 cultures grown at 55°. In addition, strain 254 contained no valine-methionine (spot 30) and with an incubation temperature of 37° only strain 295 contained spot 5 and hydroxy-proline (spot 20).

With *B. circulans*, strain 290 which formed atypical spores produced a chromatogram pattern (Table 7 and Plate 4, d) quite different from the patterns of the other 2 typical strains examined (239 and 257). It is possible that additional BMC tests would separate these organisms more clearly, although it is generally accepted by workers in this field that *B. circulans* is not an easy species to classify. Smith, Gordon & Clark (1952) experienced difficulties with this complex and made the following comment: "The present status of *B. circulans* is, therefore, extremely unsatisfactory and offers a challenge for further investigation".

The BMC characters of many of the cultures studied were atypical. Minor differences (e.g. failure to hydrolyze starch actively) were generally not reflected in the chromatogram patterns, but markedly atypical cultures invariably yielded chromatograms obviously different from those of typical cultures. With some cultures (e.g. the *B. circulans* group) the chromatogram results showed interstrain differences which were not apparent from the BMC characters. However, with the majority of cultures there seemed to be a good correlation between the chromatogram results on the one hand and the BMC characters and (to some extent) the appearances shown in electron micrographs of spores, on the other.

These preliminary experiments thus indicate a possible application of chromatography in taxonomic studies of this genus, but it is doubtful whether the method would supplant the present BMC methods for routine differentiation. However, the technique might prove of immense value in the study of the relation of thermophilic strains and new species to existing well defined species, and it is possible that chromatographic techniques of the type outlined in this paper could be usefully applied in the study of such phenomena as sporulation, heat activation and spore germination. Factors which influence the growth of micro-organisms, e.g. composition of medium, pH, temperature and degree of aeration, could be studied in greater detail and the characterization of some of the unusual ninhydrin positive materials (e.g. spot 10) should provide interesting information on bacterial metabolism.

The authors are indebted to Dr. N. J. Berridge for the interest he has shown in this work and to Miss K. V. I. Carmichael and Mr. B. A. Phillips for technical assistance.

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Explanations of Plates

Plate 1. The influence of the washing procedure on the chromatograms of the ninhydrin positive substances extracted from vegetative cells of *B. polymyxa* 237 (b,c,d) and *B. pumilus* 223 (e,f,g).

a, uninoculated medium.
b and e, culture supernatants.
c and f, first washing supernatants.
d and g, cell extracts.

Plates 2–5. Representative chromatograms.

Plate 2. (a) *B. subtilis* 4, (b) *B. licheniformis* 82, (c) *B. pumilus* 235, (d) *B. firmus* 245, (e) *B. cereus* 108, (f) *B. megaterium* 226.

Plate 3. (a) *B. lentus* 222, (b) *B. lentus* 251, (c) *B. coagulans* 295, (d) *B. coagulans* 265, (e) *B. pulvifaciens* 216.

Plate 4. (a) *B. polymyxa* 237, (b) *B. macerans* 134, (c) *B. circulans* 257, (d) *B. circulans* 290, (e) *B. alvei* 248.

Plate 5. (a) *B. brevis* 253, (b) *B. laterosporus* 219, (c) *B. pantothenticus* 233, (d) *B. sphaericus* 256, (e) *B. pasteurii* 299.

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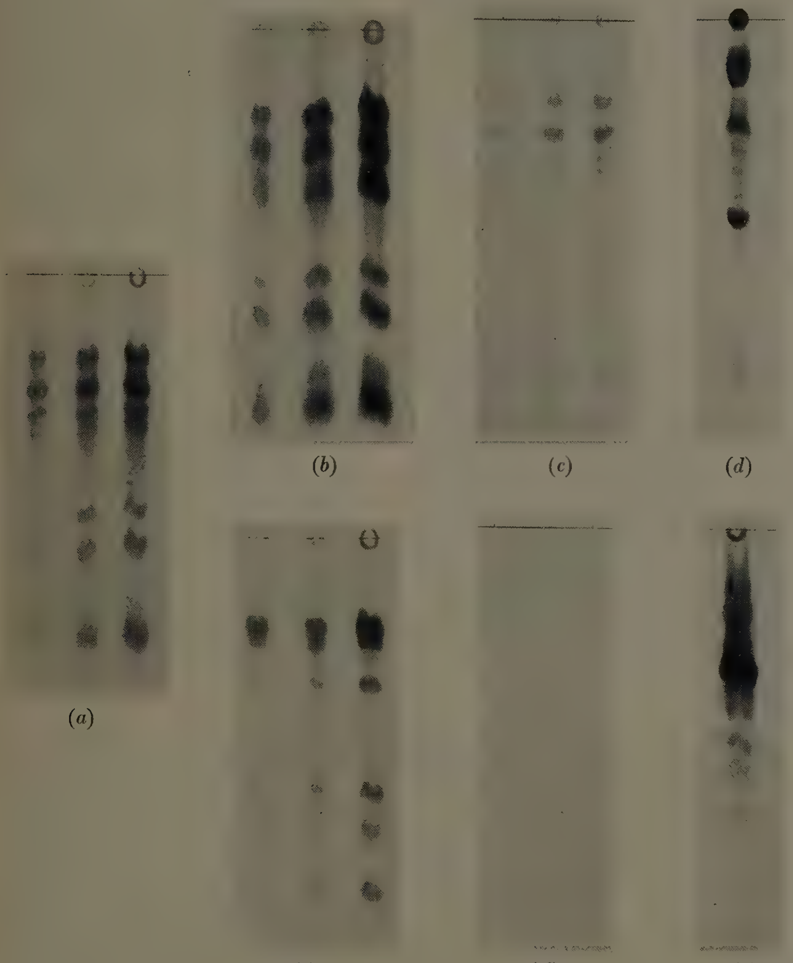
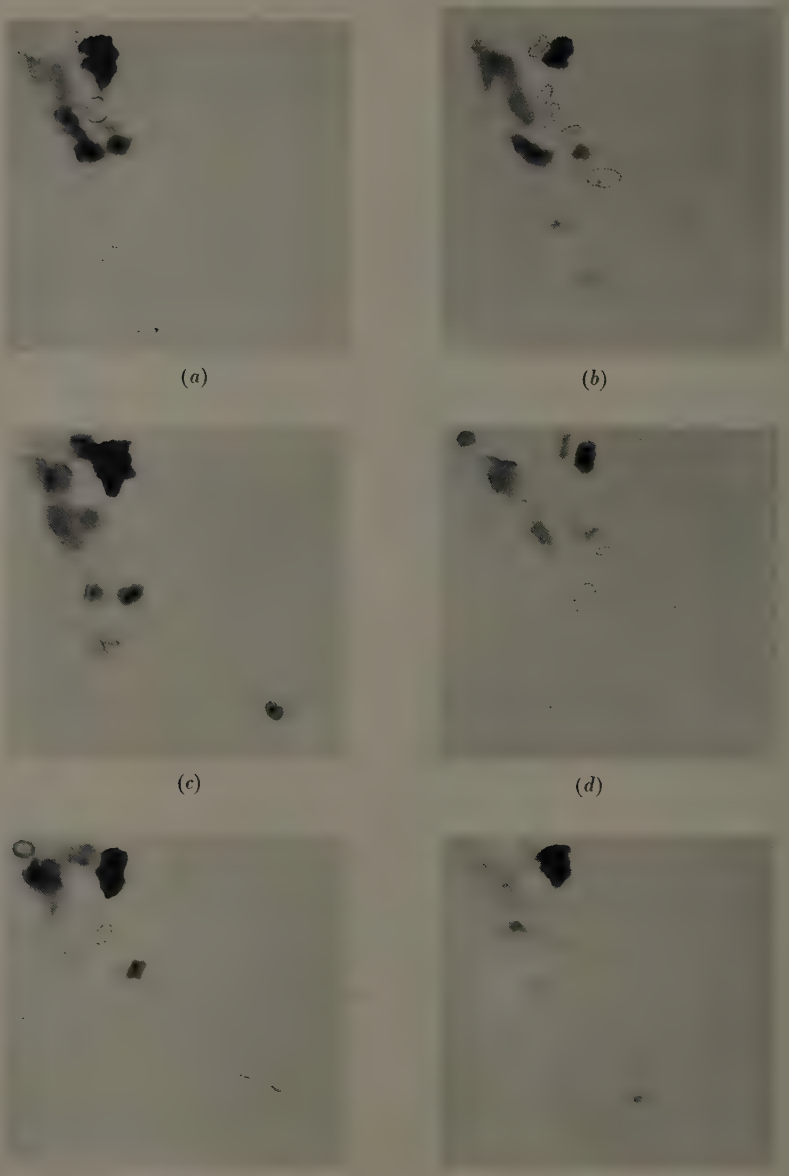

(a) (b) (c) (d) (e) (f) (g)

D. J. JAYNE-WILLIAMS & G. C. CHEESEMAN — THE DIFFERENTIATION OF BACTERIAL SPECIES BY PAPER CHROMATOGRAPHY. IX. THE GENUS *BACILLUS*: A PRELIMINARY INVESTIGATION.
PLATE 1



(*a*) (*b*)

(*c*) (*d*)

(*e*) (*f*)

D. J. Jayne Williams & G. C. Cheeseman — The Differentiation of Bacterial Species by Paper Chromatography. IX. The Genus *Bacillus*: A Preliminary Investigation.
Plate 2

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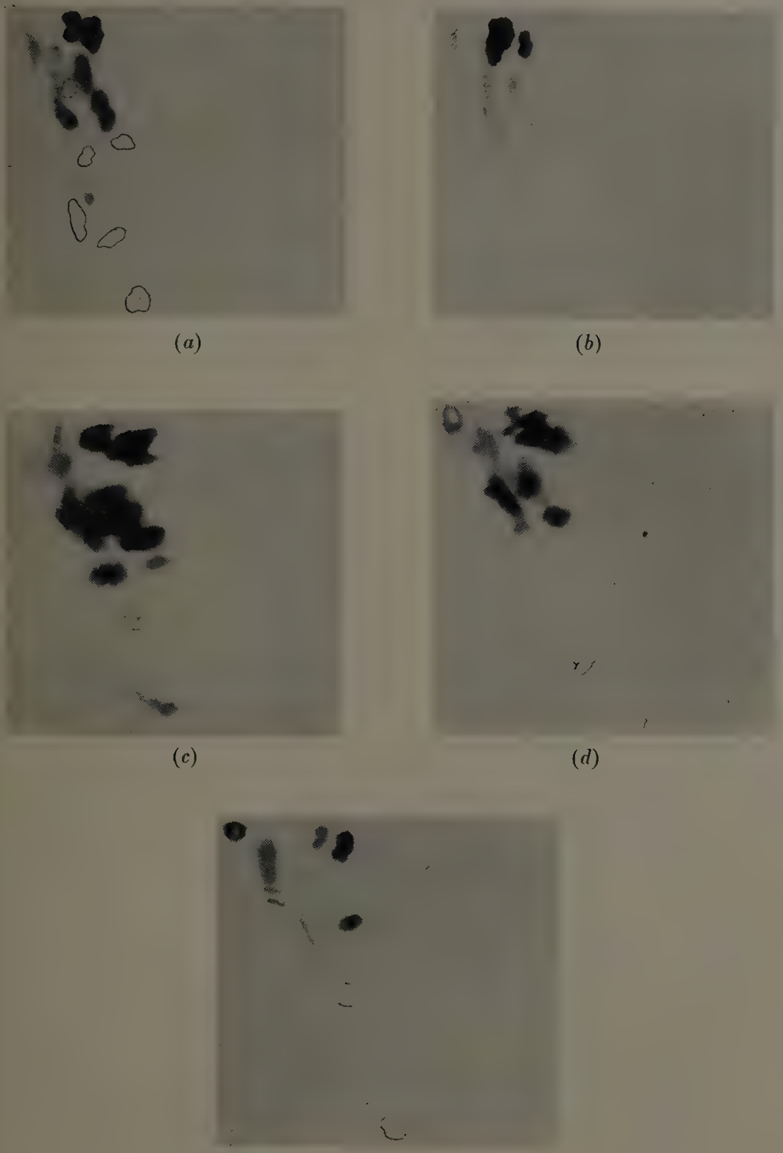
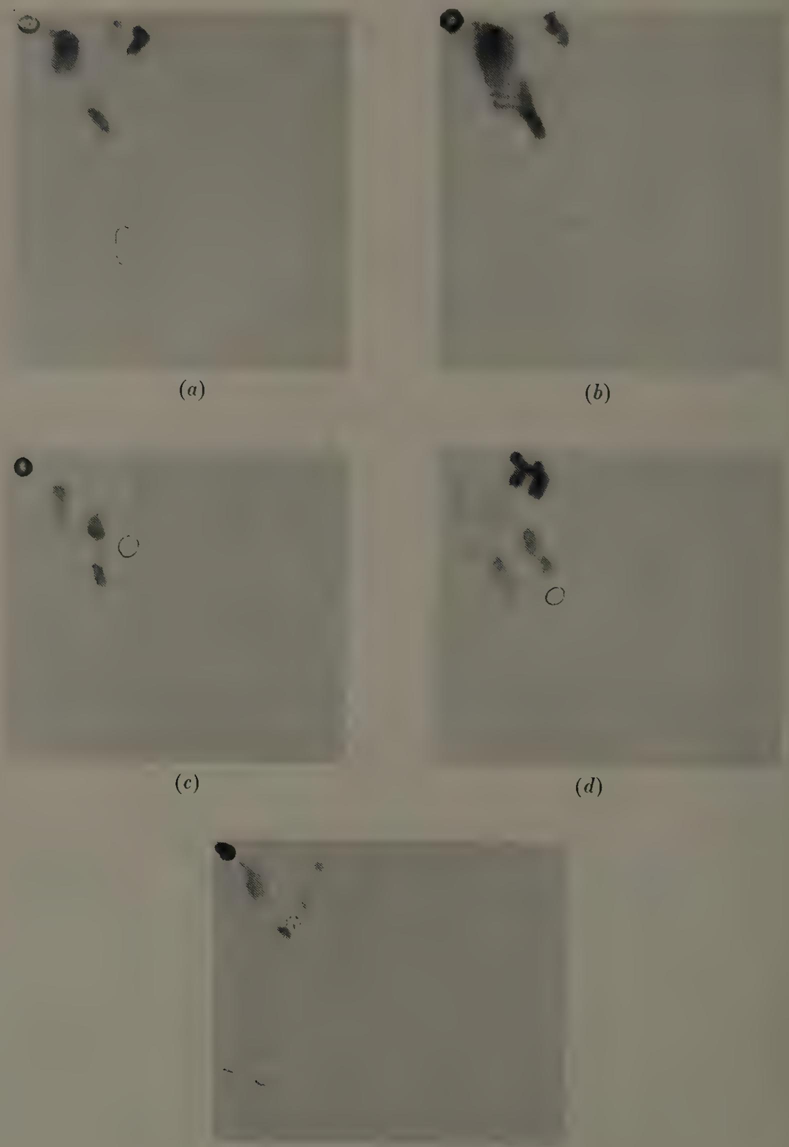


(*a*) (*b*)

(*c*) (*d*)

(*e*)

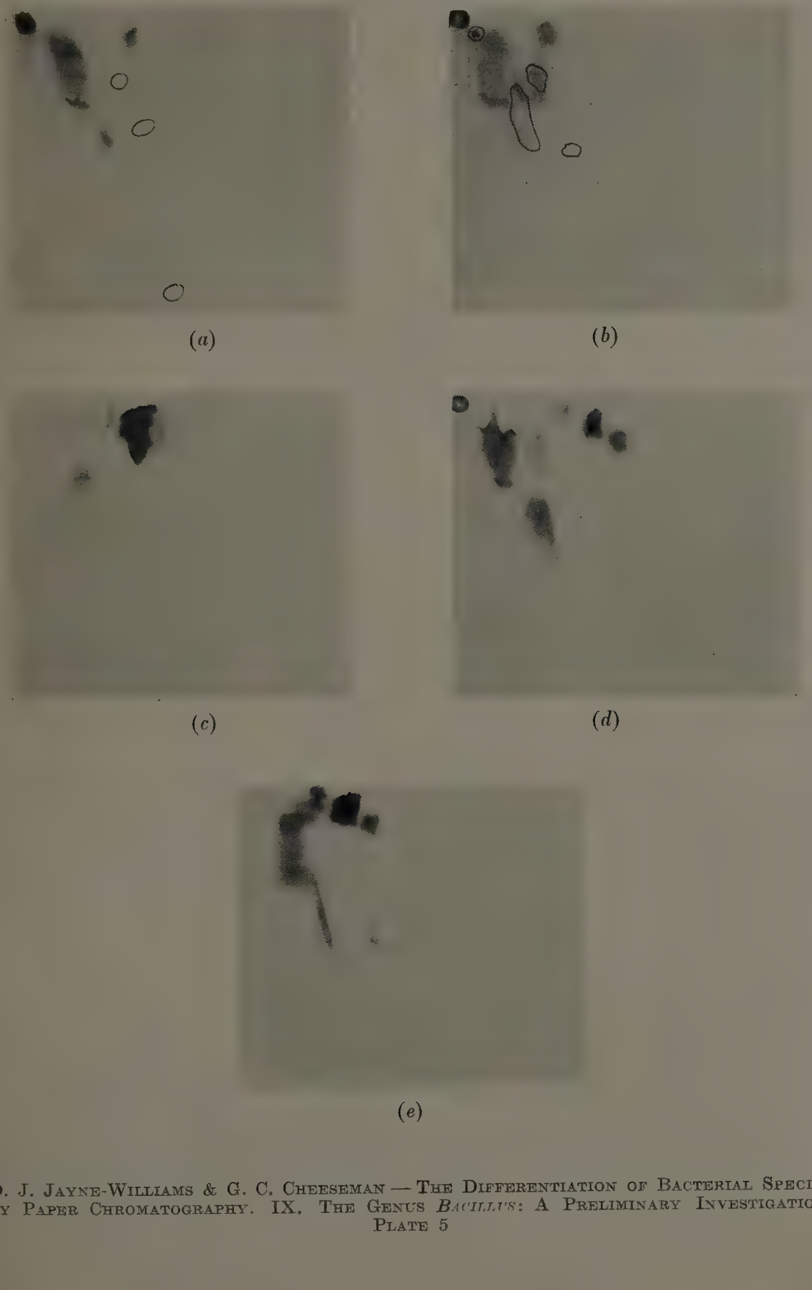
D. J. Jayne-Williams & G. C. Cheeseman — The Differentiation of Bacterial Species by Paper Chromatography. IX. The Genus *Bacillus*: A Preliminary Investigation.
Plate 3



(*a*) (*b*) (*c*) (*d*) (*e*)

D. J. Jayne-Williams & G. C. Cheeseman — The Differentiation of Bacterial Species by Paper Chromatography. IX. The Genus *Bacillus*: A Preliminary Investigation. Plate 4

Journal of Applied Bacteriology, Vol. 23, Part 2



(*a*) (*b*)

(*c*) (*d*)

(*e*)

D. J. Jayne-Williams & G. C. Cheeseman — The Differentiation of Bacterial Species by Paper Chromatography. IX. The Genus *Bacillus*: A Preliminary Investigation. Plate 5

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A METHOD FOR ESTIMATING LOW CONCENTRATIONS OF DIFFUSIBLE PENICILLINASE

By A. J. HEDGES

Department of Bacteriology, University of Bristol

SUMMARY: A method suitable for the assay of low concentrations of diffusible penicillinase has been developed and its technique is described. The concentration of soluble penicillinase in a sample is estimated by comparing its rate of diffusion in an agar gel with that of a standard preparation, the extent of diffusion being detected, after a fixed period, by a microbiological method employing 'zones of exhibition'. The method enables four similar samples to be compared on a single large plate in the form of a randomized Latin Square. Under these conditions a concentration of penicillinase able to inactivate only 2 units of penicillin/ml/hr was measurable with an experimental error (95% confidence interval) of *c.* ±10%.

When working on staphylococcal penicillinase, the author (Hedges, 1959) required to measure the low concentrations of this enzyme which appeared in the external medium after bacterial multiplication. Preliminary experiments indicated that the 'exhibition-zone' techniques, originated by Gots (1945) and modified by Broderson (1947) and Grunbach & Boyer (1948), were likely to be sufficiently sensitive and led to the development of the present method.

The principle of the 'exhibition zone' techniques is as follows. Samples of penicillinase are allowed to diffuse from suitable reservoirs into a transparent nutrient agar which has been seeded with a penicillin sensitive indicator strain in the presence of an inhibitory concentration of penicillin. After incubation the reservoirs are surrounded by zones of growth of the indicator strain ('exhibition zones') where diffusing penicillinase has sufficiently inactivated the penicillin in the medium. The size of these exhibition zones is related to the concentration of penicillinase in the reservoirs, thus allowing quantitative comparison of the sample strengths.

Materials and Methods

Assay medium. This was ox heart infusion broth, containing a final concentration of 1% (w/v) of Bacto-peptone (Difco) and 0·5% (w/v) of NaCl. The medium was buffered at pH 7·0 by means of commercial phosphate tablets (Gurr). After dispensing in amounts of 300 and 100 ml it was sterilized in the autoclave at 15 lb/in^2 for 20 min.

Assay plates. Desiccating dishes (12×12×1 in.) made from heat resisting glass (Jencons, Ltd., Hemel Hempstead) were used to contain the medium (see Simpson, 1958). Lids were made locally from sheet aluminium. The dishes, with lids *in situ*, were sterilized in the autoclave at 15 lb/in^2 for 20 min.

Indicator organism. The Mayo strain of staphylococcus (our ref: 6473), for which the minimum inhibitory concentration of penicillin is *c.* 0·05 u./ml, was used throughout these investigations. It was maintained on nutrient agar slopes at 4°, with subcultivation at roughly 6-monthly intervals. Before use in an assay a subculture was always made on a blood agar plate to check for purity. Subsequent broth cultures were inoculated with three colonies from this plate.

Diluent. The routine diluent was sterile, de-ionized water ('Elgastat', Elga Products, Ltd.) buffered at pH 7·0 with the commercial buffer tablets.

Solution of penicillin. Crystalline sodium benzyl-penicillin (Glaxo) was used. A solution containing 7·5 u./ml was made daily in the buffered diluent.

Preparations of staphylococcal extracellular penicillinase. Strains of penicillin resistant *Staphylococcus aureus*, isolated from clinical material, were grown aerobically at 37° in shallow layers of the following medium: 5% (w/v) of Bacto-peptone with 0·5% (w/v) of NaCl in distilled water, buffered at pH 7·0 with commercial buffer tablets (Hedges, 1959). After incubation for 24 hr the fluid was freed from cells by centrifugation at 2,700 ***g*** followed by filtration through a cellulose nitrate membrane of average pore diameter 0·25 μ (Membranfiltergesellschaft, supplied by Hudes Merchandising Corpn., Ltd.).

Technique

Preparation of the assay plate. One 300 ml and two 100 ml amounts of medium were used for each plate. After melting, the medium was held at 50° in a waterbath. Three ml of the solution of penicillin were added to the 300 ml quantity of medium and mixed by gentle agitation. The assay plate having been set upon a specially levelled surface, the penicillin agar was poured gently into it from a height of about 2 in. During the pouring of this 'basal layer' the plate was tilted in several directions in order to ensure even wetting of its surface. The medium was then allowed to solidify with the plate in a level position.

One ml of medium was next removed from one of the flasks containing 100 ml and replaced by 1 ml of the solution of penicillin. This flask received also 1 ml of an 18 hr nutrient broth culture of the indicator strain. After mixing by gentle agitation the contents of this flask were poured, gently as described above, over the basal layer to form an 'inoculum layer', which was allowed to solidify with the plate in a level position.

Finally, 1 ml of the solution of penicillin was added to the remaining 100 ml quantity of medium and this, after mixing, was similarly poured over the inoculum layer to form a 'top layer'.

The assay plate then contained 3 layers of medium, to a total depth of *c.* 9 mm, as follows: a basal layer *c.* 5 mm deep; an inoculum layer *c.* 2 mm deep, containing 10^6–10^7 viable cells/ml; and a top layer *c.* 2 mm deep. The concentration of penicillin was 0·074 units/ml throughout the plate. At this stage the plate was placed at 4° in the refrigerator for 30 min to harden the medium prior to cutting reservoirs and to arrest growth of the indicator strain.

Preparation of samples for assay. All samples to be assayed, if not already buffered at pH 7·0, received either buffer tablets or buffered diluent in order to maintain this hydrogen-ion concentration.

Four samples of penicillinase were compared on each plate. Whenever possible these samples were so chosen that their concentrations were roughly equal. If this was not possible, the more concentrated samples were diluted (in the buffered diluent) until they were not more than roughly twice as 'potent' as the weakest sample. When the approximate potencies of the samples were unknown a preliminary assay was done to determine the necessary degree of dilution.

From each of the four (adjusted) samples a further dilution of 1:2 was made in the buffered diluent. This resulted in a set of 8 'treatments'/plate (4 samples, each at 2 dosage levels). The minimum convenient volume of each treatment was 4 ml.

The assay procedure. The plate was removed from the refrigerator and 64 cups of 9 mm diameter were cut in the medium, using a sterile no. 5 cork borer. Positioning was facilitated by placing a rectilinear (8 × 8) grid with 27 mm between its intersections beneath the plate. Many such templates were produced by the usual office 'duplicating' methods. The grid was easily visible through the transparent medium and the cups were cut over the intersections. The plugs of medium were removed with a sterile lancet headed needle.

The 8 treatments were randomly allocated to the letters A to H and these letters were marked on the template in the form of a randomized 8 × 8 Latin Square: a fresh randomization was used for each plate. With the template in position the cups were filled as follows. A sterile Pasteur pipette (delivering *c.* 30 drops/ml) was washed thrice in the fluid of treatment A. The cup allotted to this treatment in the top row of the Latin Square was then filled almost to the brim, the number of drops delivered being noted, and the same number of drops was then delivered to each of the remaining cups allotted to treatment A, working down the rows of the Latin Square. The pipette was then washed thrice in buffered diluent and thrice in the fluid of treatment B and the cups allotted to this treatment were then charged (with the same number of drops as before) in a similar manner. This procedure was repeated until all the cups had been charged. The rate of delivery of the drops and the rhythm of operation were kept as constant as possible, and it took about 15 min to charge all the 64 cups.

Prediffusion and incubation. Immediately after charging the cups each assay plate was placed at 4° in the refrigerator and held there for 18 hr. This allowed diffusion of the enzyme into the medium but retarded the division and death of the cells of the indicator strain. The plate was then incubated at 37° for 24 hr.

Measurement of exhibition zones. The assay plate was examined by obliquely transmitted light against a matt black background; the appearance of the exhibition zones was then as shown in Plate 1. The zone diameters were measured to the nearest 0·1 mm with vernier callipers fitted with needle points. Sometimes, when adjacent cups contained high concentrations of penicillinase, the normally circular outlines of the zones were distorted owing to the additive effect of low concentrations of the enzyme which had diffused beyond the 'true' edges of the individual zones. Although this hazard was made infrequent by proper adjustment of the sample strength by preliminary dilution, all the zones were routinely measured across a diameter diagonally opposed to the major axes of the plate, i.e. along the direction of maximum distance between adjacent cups.

Analysis of the results

It was convenient to record the readings on the template used previously in the preparation of the assay plate. The response metameter (z) used in computing the results was then derived from each zone diameter by subtracting the diameter of the cup (0·9 cm) and entered on the template. The column, row and grand totals

									Row totals (z)
	A 1·52 0·62	B 1·53 0·63	C 1·62 0·72	D 1·42 0·52	E 1·43 0·53	F 1·61 0·71	G 1·51 0·61	H 1·33 0·93	4·77
	B 1·51 0·61	D 1·41 0·51	A 1·52 0·62	H 1·35 0·45	G 1·55 0·65	C 1·62 0·72	E 1·45 0·55	F 1·60 0·70	4·81
	C 1·64 0·74	H 1·36 0·46	D 1·41 0·51	B 1·55 0·65	A 1·57 0·67	G 1·57 0·67	F 1·65 0·75	E 1·44 0·54	4·99
	D 1·43 0·53	G 1·53 0·63	H 1·38 0·48	E 1·48 0·58	F 1·64 0·74	A 1·56 0·66	C 1·64 0·74	B 1·52 0·62	4·98
	E 1·45 0·55	C 1·64 0·74	F 1·65 0·75	A 1·57 0·67	H 1·38 0·48	D 1·45 0·55	B 1·54 0·64	G 1·55 0·65	5·03
	F 1·63 0·73	A 1·55 0·65	E 1·49 0·59	G 1·59 0·69	C 1·68 0·78	B 1·58 0·68	H 1·35 0·45	D 1·41 0·51	5·08
	G 1·55 0·65	E 1·48 0·58	B 1·54 0·64	F 1·64 0·74	D 1·46 0·56	H 1·34 0·44	A 1·55 0·65	C 1·62 0·72	4·98
	H 1·37 0·47	F 1·62 0·72	G 1·56 0·66	C 1·61 0·71	B 1·54 0·64	E 1·46 0·56	D 1·42 0·52	A 1·53 0·63	4·91
Column totals (z)	4·90	4·92	4·97	5·01	5·05	4·99	4·91	4·80	
								Grand total (z)	39·55

Fig. 1. Assay of four samples of staphylococcal penicillinase: template marked with results. Letters represent the coding of treatments; upper figures, external diameter of exhibition zones (cm); lower figures, values of z (cm).

Table 1. *'Treatment total' Table derived from the data of Fig.* 1

		Values for penicillinase sample				Grand totals
		I	II	III	IV	
Totals for treatments‡ at upper dosage level (a)*		F 5·84	C 5·87	B 5·11	D 4·21	21·03
Totals for treatments‡ at lower dosage level (b)*		G 5·21	A 5·17	E 4·48	H 3·66	18·52
Sample sums	(a)+(b)	11·05	11·04	9·59	7·87	39·55
Level differences	(a)−(b)	0·63	0·70	0·63	0·55	2·51
Sample means	(a+b)/16	0·690625	0·690000	0·599375	0·491875	—

* Each of the values in rows (a) and (b) represents the sum of eight replicate readings.

‡ The letters represent the code used in randomizing the treatments. Sample I was chosen as the 'standard'.

of z were also obtained and entered (a typical example is shown in Fig. 1) and an analysis of the variance of the individual readings of z about their grand mean was carried out, using the method of Finney (1952).

Table 2. *The analysis of variance of the data of* Fig. 1 *and* Table 1

Source of variation	Sum of squares	df*	Variance	F ratio‡	Significance‡
(i) Total z	0·550636	63	—	—	—
(ii) Between samples	0·427405	3	0·142468	688·25	Very highly significant
(iii) Linear regression	0·098439	1	0·098439	475·55	
(iv) Deviations from parallelism	0·000705	3	0·000235	1·14	Not significant
(v) Between treatments	0·526549	7	0·075221	363·39	Very highly significant
(vi) Between columns§	0·009999	7	0·001428	6·90	Highly significant
(vii) Between rows§	0·005389	7	0·000770	3·72	
(viii) Residual	0·008699	42	0·000207	—	—

* df, degrees of freedom.

‡ The F ratio (variance ratio) was referred to the Tables of Fisher & Yates (1957) and the 5% level of probability was chosen as the criterion for statistical significance.

§ Columns and rows of the Latin Square.

From the results of the assay 'treatment total' Tables were prepared. The data in Fig. 1 gave the 'treatment total' Table 1, and the sums of squares shown in Table 2 were computed as follows.

(i) Total z: $\sum_{i=1}^{i=64} z_i^2 - (39{\cdot}55)^2/64$

(ii) Between samples:

$$(11{\cdot}05^2+11{\cdot}04^2+9{\cdot}59^2+7{\cdot}87^2)/16 - (39{\cdot}55)^2/64$$

(iii) Linear regression: $(2{\cdot}51)^2/64$

(iv) Deviations from parallelism:

$$(0{\cdot}63^2+0{\cdot}70^2+0{\cdot}63^2+0{\cdot}55^2)/16 - \text{(iii)}$$

(v) Between treatments:

$$(5{\cdot}84^2+5{\cdot}87^2+\ldots+4{\cdot}48^2+3{\cdot}66^2)/8 - (39{\cdot}55)^2/64$$

Check: (v) = (ii) + (iii) + (iv)

(vi) Between columns:

$$(4{\cdot}90^2+4{\cdot}92^2+\ldots+4{\cdot}91^2+4{\cdot}80^2)/8 - (39{\cdot}55)^2/64$$

(vii) Between rows:

$$(4{\cdot}77^2+4{\cdot}81^2+\ldots+4{\cdot}98^2+4{\cdot}91^2)/8 - (39{\cdot}55)^2/64$$

(viii) Residual: (i) − [(v) + (vi) + (vii)]

Interpretation of the analysis of variance

The statistically highly significant difference 'between samples' in the example suggested that the samples had not been sufficiently equalized by preliminary dilution (in fact, for the purpose of illustration, no preliminary adjustment had been made). When samples differed widely in concentration estimation of their relative potency was less precise (*vide infra*).

The statistically highly significant difference 'between treatments' and the large portion of total variance accounted for by linear regression of response with respect to treatment level were considered indicative of a satisfactory assay.

The very small sum of squares due to departure from parallelism of the regression lines of individual samples was also satisfactory. Whenever a statistically significant value was obtained for this component the assay was repeated. The main reasons for the occurrence of a significant value will be discussed later.

The statistically significant components due to differences between rows and between columns of the Latin Square demonstrate the advantage of this design over methods employing separate Petri dishes, where these components cannot easily be extracted from the residual sum of squares. The main reason for the importance of these positional effects is thought to derive from temperature gradients across the plate, set up during refrigeration and incubation (see Lees & Toothill, 1955). Variation in the temperatures attained by individual Petri dishes during incubation can be even greater (Cooper & Linton, 1952).

The very small value regularly obtained for the residual variance promised a reasonable degree of precision in the estimation of the relative potencies.

Computation of relative potencies

One sample on each plate was chosen as an arbitrary standard and the concentration (or 'potency') of the other samples was estimated relative to this standard. When a large number of preparations were compared it was necessary to include the arbitrary standard on each plate.

The mean common slope ($\bar{b}$) of the regression of z on the dyadic logarithm (i.e. $\log_2$) of the treatment level was first calculated from the total level difference. The treatment levels were 'neat' ($\log_2=0$) and 1/2 ($\log_2=-1$) but, for convenience of calculation, the logarithms were 'coded' as $\log_2=+1/2$ and $\log_2=-1/2$ respectively. For the example given (Table 1) $\bar{b}=2{\cdot}51/32=0{\cdot}078438$.

The dyadic logarithm of the potency of a sample relative to that of the standard (M_2) was then obtained by dividing the difference between the sample means (Table 1) by $\bar{b}$. Multiplication of M_2 by 0·3010 converted this value into a common logarithm (M_{10}) and the relative potency (R) of the sample was then obtained as the antilogarithm of M_{10}. This value of R referred to the samples assayed and required adjustment by a factor appropriate to the preliminary dilution of the sample, if any. Table 3 sets out the computation of relative potencies in the present example. The rather wide range of the results confirmed the suggestion, obtained earlier, that these samples should have received preliminary adjustment.

Computation of confidence intervals

Upper and lower limits to include 95% of similar estimates were assigned to the values of R according to the method of Finney (1952). Results were first obtained for each of the values of M_2 from the expression

$$[M_2 \pm 2{\cdot}02\ s/\bar{b}\{(1-g)/8+M_2^2/16\}^{1/2}]/(1-g),$$

where $g=(4{\cdot}0804\times s^2)/(16\times \bar{b}^2)$ and s^2 is the residual variance (Table 2).

The limits so obtained were themselves dyadic logarithms and were therefore converted to common logarithms before taking antilogarithms to give the upper (R_U) and lower (R_L) limits of R. These limits are set out for each of the samples of the present example in Table 4.

Table 3. *The computation of the relative potencies of samples from the data of* Table 1

Sample	Log. of relative potency (relative to sample I)		Relative potency (R)
	Dyadic (M_2)	Common (M_{10})	
I	0	0	1·000
II	$\frac{-0{\cdot}000625}{0{\cdot}078438} = -0{\cdot}007968$	$\bar{1}{\cdot}9976$	0·995
III	$\frac{-0{\cdot}091250}{0{\cdot}078438} = -1{\cdot}163339$	$\bar{1}{\cdot}6498$	0·447
IV	$\frac{-0{\cdot}198750}{0{\cdot}078438} = -2{\cdot}533848$	$\bar{1}{\cdot}2373$	0·173

Table 4. *The* 95% *confidence intervals for the estimated relative potencies of* Table 4

Sample	Relative potency (R)	Limits of intervals	
		Upper (R_U)	Lower (R_L)
I	1·000	—	—
II	0·995	1·089 +9·4%*	0·908 −8·7%*
III	0·447	0·499 +11·6%*	0·394 −11·9%*
IV	0·173	0·205 +18·5%*	0·141 −18·5%*

* Difference between limit and relative potency, as a percentage of the estimated potency.

It is at once evident that the width of the confidence interval was minimal when the samples under comparison were of similar potency. This property constituted one of the chief reasons for the preliminary equalization of sample strengths. The figures given in the example are typical of those obtained in many similar assays and it is clear that the 'experimental error' of the method (95% confidence interval) is *c.* ±10% when the samples are sufficiently similar in potency.

RESULTS

Investigation of factors affecting the technique

The three-layered plate. Preliminary experiments showed that when the indicator strain was present throughout the medium the edges of the exhibition zones were less distinct than when the inoculum was restricted to a single layer. The basal layer was poured merely to increase the total depth of medium and, therefore, the volume of the reservoirs. The function of the top layer was twofold: it protected the inoculum layer from mechanical damage when the cups were cut and prevented the displacement of cells of the indicator strain in water condensing upon the surface of the medium.

Density of inoculum and concentration of penicillin. The definition of the zone edge was maximal when the density of viable cells in the inoculum layer was 10^6–10^7/ml. Higher densities gave rise to exhibition zones with 'fuzzy' edges; with lighter inocula the colonies were too sparse for accurate measurement. Previous experience in this laboratory has shown that the optimum density was regularly obtained by making a final 10^{-2} dilution of an overnight broth culture of the indicator strain.

The concentration of penicillin affected both the mean size of the exhibition zones and the value of $\bar{b}$, and these both became maximal when it was only slightly greater than the minimum inhibitory concentration for the indicator strain (Table 5). Because the sensitivity of the method was favoured by producing large zones with the lowest concentrations of penicillinase, and because the precision of estimation was favoured, *ceteris paribus*, by large values of $\bar{b}$, the lowest workable level of penicillin was used. This was *c.* 0·075 u./ml, since at lower concentrations some colonies appeared outside the exhibition zones.

Table 5. *The effect of the concentration of penicillin in the assay medium on the mean value of z and the mean slope of the regression line**

Penicillin concn. (u./ml.)	Mean values of z (cm)	Mean values of slope ($\bar{b}$)
0·050	1·70	0·60
0·075	1·28	0·50
0·100	1·07	0·30
0·125	0·88	0·35

* Data from an assay of *B. licheniformis* penicillinase (commercial, Burroughs Wellcome). The same three concentrations of penicillinase were used at each level of penicillin.

Hydrogen ion concentration. Since most penicillinases are reported to show maximum activity at pH values around 7·0 (see Abraham, 1951; Hedges, 1959) all preparations and media used were buffered at that level.

Prediffusion. By allowing the penicillinase to diffuse from the reservoirs under conditions (i.e. refrigeration) that precluded cell division both the mean size of the exhibition zones and the value of $\bar{b}$ were increased. When the period of prediffusion exceeded 24 hr the regression of z on log. concentration of penicillinase began to depart seriously from linearity, presumably owing to the significant lowering of the concentration of penicillinase in the reservoirs following prolonged diffusion. A period of 18 hr was therefore chosen as affording the best compromise between maximal sensitivity and precision.

Period of incubation. A period of 24 hr at 37° was chosen because the exhibition zones did not appreciably increase in size during the following 7 days, while the zone edge often became less distinct.

Order of charging the cups. The time taken to charge all 64 cups on an assay plate was about 15 min. Those who have used large plates in the assay of antibiotics have found that significant diffusion often occurs during the charging of the reservoirs (Brownlee *et al.* 1948; Lees & Toothill, 1955). In contrast, the rate of diffusion of penicillinase was so slow that no difference could be detected between the responses around cups charged first and last. It was therefore unnecessary to specify any special order of charging.

Volume of fluid placed in the cups. Experiment showed that it was important to deliver a constant volume of fluid to each cup. When the cups were 'filled to the brim', using a different Pasteur pipette for each sample, identical samples appeared to differ in potency, sometimes by as much as 23%; but when a constant volume was delivered to each cup no significant difference was detected between identical samples. Recent experiments have shown that the somewhat tedious dropping method may be satisfactorily replaced by the use of a semi-automatic constant volume pipette (H. G. East & Co., Ltd., Oxford) set to deliver 0·4 ml.

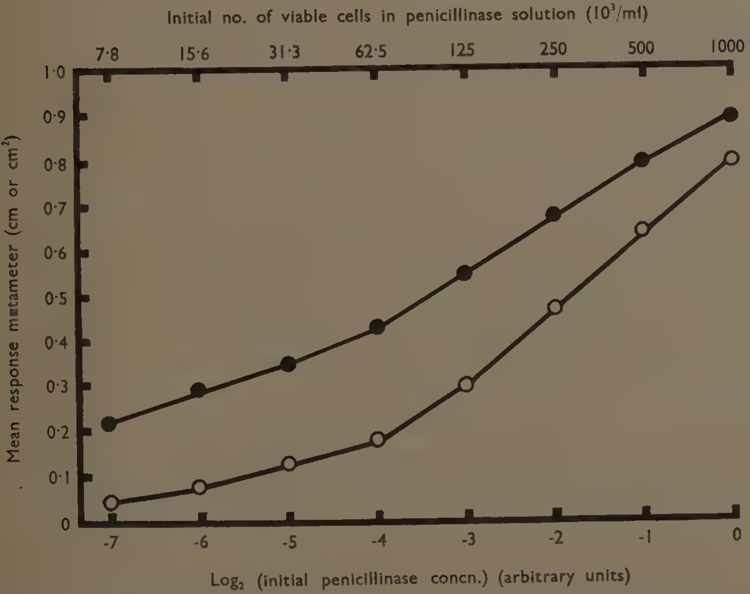

Fig. 2. The regressions of two response metameters on $\log_2$ (concentration of non-sterile penicillinase solution). Full circles, z (means of 8 readings); open circles, z^2 (means of 8 values).

The presence of penicillinase producing cells in the fluids to be assayed. In many of the studies for which the method was required it was inconvenient to pass all the samples through a bacterial filter. Some samples were therefore assayed after centrifugation but without filtration. When a mature culture was so treated the viable cell density in the supernatant fluid was of the order of 10^6/ml, and the question arose, whether these residual cells could affect the results of the assay.

Evidence obtained from a number of experiments suggested that the residual cells could form additional extracellular penicillinase in the reservoirs during the incubation of the assay plate, thereby increasing the size of the exhibition zones. However, this effect was important only when either the period of prediffusion was curtailed or omitted, or the initial concentration of extracellular penicillinase in the sample was low (cf. Figs. 2 and 3). It was evident that, for the highest accuracy, the fluids to be assayed should not contain viable cells; for less accurate estimations filtration could be omitted when the initial concentration of extracellular penicillinase was high (e.g. in mature cultures).

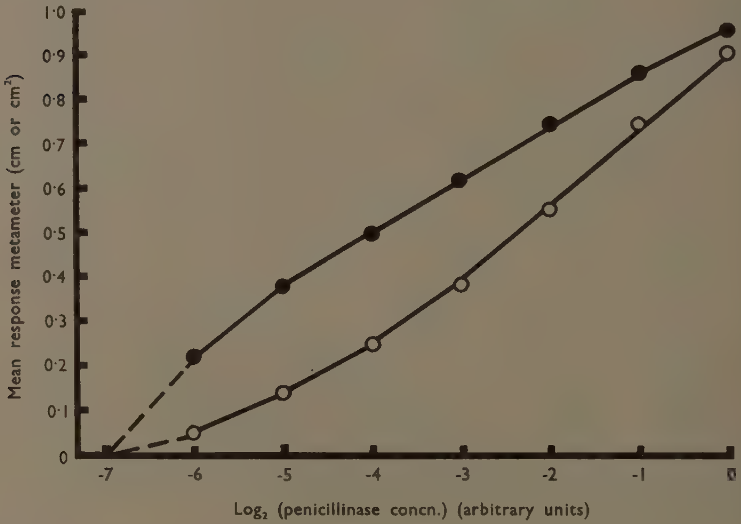

Fig. 3. The regressions of two response metameters on $\log_2$ (concentration of sterile penicillinase solution). Full circles, z (means of 8 readings); open circles, z^2 (means of 8 values).

Dilution and manipulation of samples. No difference was detected when nutrient medium was substituted for the buffered diluent. It was therefore concluded that the size of the exhibition zones was unaffected by nutrients diffusing from the cups.

Of obvious importance was the finding that the manipulation of samples, in preparing dilutions, could be responsible for an appreciable lowering of their activity. Apart from denaturation of the enzyme on the surface of a foam, it appeared that adsorption to the glass surface of a pipette might be responsible for part of the effect. For this reason all pipettes were thoroughly wetted with the sample before beginning the manipulation.

When dilutions were 'mixed' by repeated aspiration and expulsion (5 times) of the fluid, using a glass 10 ml graduated pipette, inactivation to the extent of *c.* 15% was regularly recorded. Later preparations of the enzyme were therefore mixed by gentle agitation.

Investigation of factors concerning the analysis of the assay results

Choice of dose and response metameters. When z and z^2 were plotted against the logarithms of the concentration of penicillinase placed in the cups, neither metameter showed a truly linear regression over the whole range of readings (Fig. 3). However, both metameters approximated to linear functions of log. concentration over a range sufficient to allow their use in the type of assay described. In the absence of any theoretical preference for either metameter (Hedges, 1959) the quantity z was chosen to represent the response for the two reasons of ease of computation and homogeneity of variance. Bartlett's test (see Finney, 1952) was applied, in turn, to the variances of z and z^2 about their array means over the range of values shown in Fig. 3. Whereas the distribution of z^2 was markedly heteroscedastic ($\chi^2_{[6]} = 19{\cdot}8$; $P < 0{\cdot}01$), the variance of z was reasonably constant over the whole range examined ($\chi^2_{[6]} = 6{\cdot}7$; $P > 0{\cdot}1$).

The choice of the dilution ratio between the upper and lower treatment levels depended on whether significantly different responses at the two levels could be obtained without the range being so wide as to exceed the limits for linearity of the regression. A ratio of 1:2 proved satisfactory and the dyadic logarithms, $+1/2$ and $-1/2$, were therefore chosen to represent the upper and lower levels respectively.

Check for parallelism of individual regression lines. The regression lines for different samples deviated significantly from parallelism under two sets of conditions. First, when the sample strengths differed so widely that the responses extended over non-linear sections of the graph (Fig. 3) a statistically significant component was obtained in the analysis of variance. This hazard was easily reduced by the preliminary adjustment of samples described previously.

The second source of deviation from parallelism was encountered in the comparison of penicillinases from different bacterial species. When a staphylococcal extracellular penicillinase was compared with a commercial preparation from *Bacillus licheniformis* (Burroughs Wellcome) the regression lines were markedly nonparallel. In contrast, the regression lines for preparations of extracellular penicillinase obtained from many different strains of *Staphylococcus aureus* were always parallel within the experimental error. The lack of parallelism in the comparison of samples from the different organisms suggests a difference in the molecular structure of their penicillinases. A similar experience has been recorded by Broderson (1947).

DISCUSSION

Since its discovery in a strain of *Bacterium coli* by Abraham & Chain (1940) many different properties of penicillinase have received attention (for detailed reviews see Abraham, 1951; Elek, 1959; Hedges, 1959). Fundamental to any quantitative study of the enzyme is the necessity of a reliable method of estimation. A number of methods have been published and these may be put into four groups.

(a) Serial dilution of samples to give a predetermined titre (Harper, 1943; Kirby, 1944; Housewright & Henry, 1947; and many others).

(b) Estimation of the penicillin remaining intact after reacting the samples with a known quantity under standardized conditions (Abraham & Chain, 1940; Bondi & Dietz, 1944; Woodruff & Foster, 1945; Gilson & Parker, 1948; Eriksen & Hansen, 1954; and many others).

(c) Estimation of the rate of formation of penicilloic acid from penicillin by the action of the penicillinase in the samples under standardized conditions, using the alkalimetric method of Wise & Twigg (1950), the manometric method of Henry & Housewright (1947), or iodometric methods (Pénau, Phillipe & Benoist, 1951; Tucker, 1954; Perret, 1954).

(d) Estimation of the concentration of penicillinase by its rate of diffusion through an agar gel, i.e. the 'exhibition zone' methods.

Each group of methods has its peculiar advantages and disadvantages for specific requirements. The methods of group (a) are very sensitive but, unless their techniques are elaborated to an extent when they become tedious, their precision is low. Similar remarks apply also to the methods of group (b), where the errors of the secondary assay of penicillin are additive to the variation in the extent of hydrolysis. The chemical methods of group (c) are capable of high precision but, except perhaps for the iodometric methods, they are relatively insensitive to low concentrations of the enzyme. In contrast, the exhibition zone methods possess the high sensitivity usually associated with microbiological methods; but they share also their generally wide variation.

The yields of extracellular penicillinase obtained from strains of *Staph. aureus* are generally very low; in fact several authors have failed to detect the enzyme in the culture fluids of penicillinase producing strains (Spink & Ferris, 1947; Geronimus & Cohen, 1957). For this reason the exhibition zone methods seemed the most promising for assaying staphylococcal extracellular penicillinase provided the precision of estimation could be increased. By adjusting a number of variables to their optima, and by using a design of the Latin Square form, the 95% confidence interval for the estimation of the relative potency of similar samples was reduced to *c.* ±10%. The sensitivity of the method may be judged from the result that samples of penicillinase were assayed whose potency was such that, under the conditions necessary for a zero-order reaction, only 2 units of penicillin were hydrolyzed/ml/hr (method of Tucker, 1954).

The general application of the present method is limited by a number of considerations. First, only true solutions of penicillinase may be assayed: this excludes the majority of preparations of intracellular enzyme, which are suspensions of particulate material. Second, samples must not contain diffusible substances which are inhibitory to the indicator strain. Third, samples must be adjusted to, and buffered at, the same pH, otherwise the activity of the enzyme will be differentially affected; and fourth, the day to day variation experienced in the values of mean zone diameter and of $\bar{b}$, although small, is such that a 'standard' preparation of the enzyme must be included on each assay plate.

In spite of these limitations, the method was well suited to making comparative estimations of low concentrations of staphylococcal extracellular penicillinase, and it seems reasonable to suppose that the method may find application in similar studies on soluble penicillinases produced by other micro-organisms.

I am indebted to Prof. K. E. Cooper whose friendly criticism and stimulating advice were of great value throughout these investigations. My thanks are due also to Dr. G. Herdan for advice on certain of the mathematical statistics and to Miss B. G. Waters for her expert preparation of the nutrient media.

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EXPLANATION OF PLATE

A penicillinase assay plate, showing the appearance of the exhibition zones at the time of measurement (× ½).

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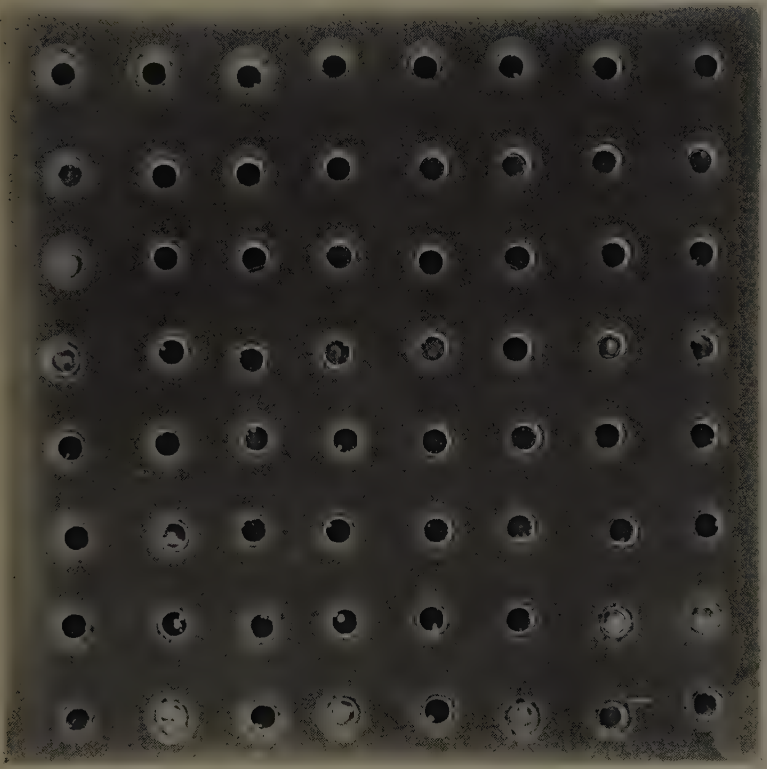

HEDGES, A. J. — A METHOD FOR ESTIMATING LOW CONCENTRATIONS OF DIFFUSIBLE PENICILLINASE. PLATE 1

HOPTON, J. W. & WOODBINE, M. (1960). *J. appl. Bact.* **23** (2), 283–290.

FAT SYNTHESIS BY YEASTS. I. A COMPARATIVE ASSESSMENT OF *HANSENULA* SPECIES

BY J. W. HOPTON AND M. WOODBINE

Microbiological Unit, Department of Agricultural Sciences,
University of Nottingham

SUMMARY: A comparative examination of the growth of 15 species of *Hansenula* in typical 'fat' media has been undertaken. Seven species showed a good yield on the basis of sugar consumed and, in terms of fat content, they fell into three broad categories. *H. ciferrii* and *H. saturnus* had a fat content (*c.* 20%) rather lower than that of *Rhodotorula gracilis* grown under the same conditions; 2 strains of *H. anomala* had a fat content of about 13%, and *H. jadinii*, *H. angusta* and *H. subpelliculosa* had a relatively low fat content (*c.* 8%). In a 'fat' medium containing urea as principal nitrogen source there was an initial phase during which most of the nitrogen was assimilated, followed by a phase during which most of the fat accretion occurred.

BECAUSE of the pressing need to increase world food production much attention has been paid in recent years to the possibility of utilizing micro-organisms in bulk as food supplements. In a recent review Woodbine (1959) dealt at length with one aspect of this field of investigation, namely that of microbiological fat production. He pointed out that although many microbial species have been studied for fat-producing ability, so far as the yeasts are concerned the choice has been somewhat arbitrary and only species of the genus *Rhodotorula* have received a comparative examination (Enebo *et al.* 1944). A systematic screening of more organisms is one obvious approach to the problem of selection for high fat-producing potential, and comparative analysis at the generic level has much to commend it. For this reason selected species of the genus *Hansenula* were obtained and their growth and yield in typical 'fat' media (i.e. media of high carbohydrate: nitrogen ratio) examined. A preliminary report of this work has already appeared (Hopton & Woodbine, 1958). This genus was chosen because little is known of the synthetic capabilities of its members, but Wickerham (1951) has compiled much useful information concerning the fermentative and assimilatory properties and growth requirements of a number of species.

MATERIALS AND METHODS

Yeast species. The *Hansenula* species used which all originated from the collection at the Northern Region Research Laboratory, Peoria, Ill., U.S.A., are listed in Table 1. In addition one strain of *Rhodotorula gracilis* was studied. Cultures were carried on beer wort agar and subcultured at intervals of approximately one month.

Media. Two types of basal media were employed. The first, a mineral salts-yeast extract medium (MYE) (Pan, Andreason & Kolachov, 1949) had the following composition/litre: KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 1 g; NaCl, 0·5 g; $CaCl_2.6H_2O$, 0·5 g;

$FeCl_3.7H_2O$, 0·005 g; yeast extract, 1·5 g. To this was added glucose, 40 or 60 g and a nitrogen source, in the form of $(NH_4)_2SO_4$, 1 g/l., or asparagine or urea, each in amounts which gave the same nitrogen content in the medium as the $(NH_4)_2SO_4$, i.e. 0·21 g nitrogen/l. A solution containing the minerals and yeast extract and solutions of the nitrogen source and the glucose, all at three times the concentration required in the complete medium, were sterilized separately and then combined.

Table 1. *The* Hansenula *spp. studied, and their designations*

Species	NRRL* no.	Species	NRRL* no.
H. anomala	Y-365	*H. silvicola*	Y-1678
H. anomala	Y-366	*H. californica*	Y-1680
H. minuta	Y-411	*H. subpelliculosa*	Y-1683
H. ciferrii	Y-1031	*H. suaveolens*	Y-1725
H. saturnus	Y-1304	*H. angusta*	Y-1798
H. mrakii	Y-1364	*H. capsulata*	Y-1842
H. beckii	Y-1482	*H. canadensis*	Y-1888
H. jadinii	Y-1542		

* NRRL, Northern Regional Research Laboratory.

The second basal medium was prepared from molasses. Cane molasses, having a sugar concentration (as glucose) of 58% (w/w), was treated as described by Pan *et al.* (1949) and then diluted to give a medium containing approximately 40 g of sugar (as glucose)/l. Maximum sugar utilization in this medium did not exceed 88% of the original. In two experiments this medium was supplemented by the inclusion of $(NH_4)_2SO_4$ and KH_2PO_4, each at a final concentration of 1 g/l.

The initial pH of all media was in the range 4·9–5·2.

Dispensation and inoculation of media. The complete media were distributed aseptically in 50 ml amounts in 250 ml conical flasks. For the preliminary survey (Table 2) and for the bulk cultivation of yeasts (Table 3) each flask was given an inoculum of 0·5 ml of a culture grown for 72 hr in a medium containing 3 g of malt extract, 5 g of peptone, 3 g of yeast extract and 10 g of glucose/l.

For the quantitative studies of culture development (Fig. 1 and Table 4), the inoculum culture was grown for 72 hr in MYE containing 4 g of asparagine and 40 g of glucose/l, the yeast recovered by centrifuging, washed once with distilled water, again centrifuged and then suspended in distilled water to give a dry weight concentration of 4 mg/ml. Each flask of medium was inoculated with 0·5 ml of suspension.

Cultural conditions. The flasks were agitated on a rotary shaker during incubation at 26·5±1·0°.

Analytical methods

Duplicate flasks of culture were analyzed at each sampling. The yield of yeast, sugar content and pH of the duplicates were determined separately. Agreement was invariably good and the results given are the means of the determinations. For fat and nitrogen determinations the crops of powdered dried yeast from the duplicate cultures were combined and duplicate samples were analyzed.

Yeast yield. The cell mass was recovered by centrifuging, then washed once with distilled water and centrifuged again. The resulting yeast pad was dried overnight at 80°.

Glucose estimation. The supernatant culture fluid remaining after the yeast cells had been harvested was analyzed by the method of Hulme & Narain (1931). With molasses media the fluid was first hydrolyzed with 0·35 N HCl (Pan *et al.* 1949) and the hydrolysate neutralized with N NaOH.

Nitrogen. This was determined by the method of Ling (1942).

Fat estimation. The dried yeast was first ground to a fine powder in a small coffee grinder and 0·2–1·0 g refluxed with 20 ml of N HCl for 2 hr, after which the mixture was filtered directly through an extraction thimble and the residue washed thoroughly with water. Most of the water absorbed in the thimble was removed under reduced pressure and the remainder in a vacuum oven. When 1 g samples were taken a conventional Soxhlet extractor was employed; but when the analysis of large numbers of small samples was required the extractor unit described by Wix & Hopton (1957) was used. The solvent was light petroleum (40–60°).

RESULTS

Preliminary survey of yeast yield in the various media

The results of a series of experiments in which yeast yield and sugar consumption in various media were determined are given in Table 2. Samples were taken after 3 and 5 days and sometimes after 6 or 7 days incubation. Except when unsupplemented media were used, the heaviest crops were usually obtained at the first

Table 2. *A comparison of the data for maximum sugar utilization and maximum yeast yield in different media obtained with* Hansenula *spp. and* Rhodotorula gracilis

Yeast species	Data from MYE medium with nitrogen added as:								Data from molasses with:			
	$(NH_4)_2SO_4$		Asparagine		Urea		No addition		$K_2HPO_4+(NH_4)_2SO_4$		No addition	
	Glucose used* (g)†	Yeast yield* (mg)	Glucose used* (g)†	Yeast yield* (mg)	Glucose used* (g)†	Yeast yield* (mg)	Glucose used* (g)†	Yeast yield* (mg)	Glucose used* (g)†	Yeast yield* (mg)	Glucose used* (g)†	Yeast yield* (mg)
H. anomala Y-365	1·65 (5)	144 (5)	2·09 (3)	449 (3)	2·26 (3)	598 (3)	2·10 (5)	168 (5)	1·68 (5)	459 (5)	0·96 (5)	158 (5)
H. anomala Y-366	2·08 (3)	397 (3)	2·09 (3)	610 (3)	2·26 (3)	702 (3)	2·12 (7)	320 (7)	1·70 (3)	586 (3)	1·56 (5)	258 (5)
H. minuta§	1·98 (5)	162 (3)	2·02 (5)	158 (3)	2·21 (5)	232 (5)	1·87 (7)	180 (5)	0·62 (3)	222 (3)	0·65 (5)	125 (5)
H. ciferrii	1·09 (3)	130 (3)	2·09 (3)	638 (3)	2·26 (3)	663 (3)	2·10 (7)	254 (7)	1·71 (3)	636 (3)	0·96 (5)	246 (5)
H. saturnus	1·41 (5)	321 (5)	2·09 (3)	694 (3)	2·26 (3)	626 (3)	2·10 (5)	269 (5)	1·69 (3)	644 (3)	1·27 (5)	371 (5)
H. mrakii§	1·47 (3)	407 (3)	2·09 (3)	630 (3)	2·26 (3)	662 (3)	1·38 (7)	263 (7)	0·64 (3)	259 (3)	0·65 (5)	173 (5)
H. beckii	—	—	1·85 (5)	244 (5)	1·96 (5)	237 (5)	1·83 (7)	187 (7)	1·64 (3)	696 (3)	1·61 (5)	317 (5)
H. jadinii	2·08 (3)	463 (3)	2·09 (3)	553 (3)	2·26 (3)	576 (3)	1·48 (7)	218 (7)	1·71 (3)	528 (3)	1·22 (5)	262 (5)
H. silvicola	1·51 (5)	250 (5)	2·09 (3)	220 (7)	2·26 (3)	213 (5)	1·11 (7)	125 (7)	1·09 (5)	394 (5)	0·65 (5)	143 (5)
H. californica§	—	—	2·09 (5)	501 (5)	2·26 (5)	454 (5)	0·57 (7)	129 (7)	0·72 (5)	292 (5)	0·70 (5)	185 (5)
H. subpelliculosa	2·08 (3)	495 (3)	2·09 (3)	649 (3)	2·26 (3)	674 (3)	2·10 (5)	254 (5)	1·68 (3)	679 (3)	1·54 (5)	214 (5)
H. suaveolens	1·42 (5)	299 (5)	2·09 (3)	359 (3)	2·26 (3)	358 (3)	2·10 (7)	276 (5)	1·64 (3)	479 (3)	1·13 (5)	201 (5)
H. angusta	2·08 (5)	365 (3)	1·97 (5)	499 (5)	2·26 (5)	565 (5)	2·12 (7)	222 (7)	1·65 (5)	598 (5)	0·84 (5)	259 (5)
H. capsulata§	1·95 (5)	176 (5)	2·09 (5)	346 (5)	2·26 (5)	328 (5)	2·09 (7)	274 (7)	0·71 (5)	262 (5)	0·63 (3)	171 (3)
H. canadensis	2·00 (5)	356 (5)	2·09 (5)	365 (3)	2·26 (5)	413 (5)	1·33 (7)	176 (7)	1·66 (5)	631 (3)	0·94 (5)	229 (5)
Rh. gracilis	1·54 (5)	356 (3)	1·83 (5)	520 (5)	2·13 (5)	512 (5)	0·79 (7)	191 (5)	1·71 (5)	448 (5)	0·62 (5)	134 (5)

* Maximum amounts, in the period in days shown by the bracketed numbers, for 50 ml cultures. † Initial weights (g) of glucose available:—in MYE with $(NH_4)_2SO_4$, 2·08; with asparagine, 2·09; with urea, 2·26; with no addition, 2·13; in molasses with $K_2HPO_4+(NH_4)_2SO_4$, 1·97; with no addition, 1·92. § Sucrose not assimilated.

harvesting; but in a few instances the crop was heaviest at a later stage. In MYE containing $(NH_4)_2SO_4$ the pH in all cases dropped to a low value (*c.* 2·1) by the third day and remained low. The pH fall in MYE containing urea or asparagine was less pronounced (lowest pH values *c.* 3·0) and in addition the yields from these media were, in general, higher than those from the medium supplemented with $(NH_4)_2SO_4$. This suggests that the low pH reached in $(NH_4)_2SO_4$–MYE was detrimental to cellular synthesis, and support for this is provided by the finding that in the supplemented molasses medium, where $(NH_4)_2SO_4$ was also present, the pH remained fairly steady and good yields were obtained. The yields, based on glucose consumed, were invariably low, particularly those from the two basal media, and in a number of cases the analyses showed that between successive harvestings considerable breakdown had occurred with little or no increase in cell mass.

Fat content of selected yeasts

Seven *Hansenula* yeasts which had shown the highest general yields in the preliminary survey, and *Rh. gracilis*, were grown in quantity in MYE supplemented with $(NH_4)_2SO_4$ or urea and in molasses medium supplemented with $(NH_4)_2SO_4$ and KH_2PO_4. A further species, *H. mrakii*, which had also shown a promising yield in the media containing glucose, was excluded from this more detailed examination because it was unable to assimilate sucrose. The crops were harvested at the time of optimum yield as determined in the preliminary survey (3 or 5 days), and the results are shown in Table 3.

Table 3. *A comparison of data for maximum glucose utilization* (A), *yeast yield* (B) *and fat content* (C) *in different media*, for selected* Hansenula *spp. and* Rhodotorula gracilis

Yeast species	Glucose utilization, yeast yield and fat content in								
	MYE medium with:						Molasses medium with:		
	$(NH_4)_2SO_4$			Urea			$(NH_4)_2SO_4+KH_2PO_4$		
	A†	B§	C‡	A†	B§	C‡	A†	B§	C‡
H. anomala Y-365	67	13	9·0	100	26	17·2	84	25	13·3
H. anomala Y-366	100	20	12·9	99	35	12·6	84	30	14·4
H. ciferrii	60	11	6·5	99	28	17·0	85	33	21·5
H. saturnus	74	19	18·8	91	23	20·6	65	27	20·1
H. jadinii	97	22	6·3	99	34	9·5	85	29	10·6
H. subpelliculosa	98	26	7·2	99	31	7·0	85	43	7·8
H. angusta	94	19	4·2	81	31	4·3	83	34	7·5
Rh. gracilis	38	35	29·7	87	30	22·5	86	26	29·2

* Initial glucose concentration (or sugar as glucose, for the molasses medium), *c.* 40 g/l.
† As % of initial. § Based on glucose consumed, %. ‡ %.

Sugar consumption and yield determinations confirmed, in general, the results of the preliminary survey. The fat analyses revealed that the *Hansenula* yeasts fell into three broad categories, *H. ciferrii* and *H. saturnus* being 'high fat' yeasts with a fat content comparable to that of *Rh. gracilis*, the strains of *H. anomala* 'intermediate' and *H. jadinii*, *H. angusta* and *H. subpelliculosa* 'low fat' species.

Quantitative studies of culture development

A closer comparative examination of the development of representatives of the three contrasting groups was next undertaken. The medium was MYE with urea and glucose at 40 or 60 g/l. Samples were taken at intervals during fermentation periods of 113 to 161 hr. In Fig. 1 the results for a 'high fat' yeast (*H. saturnus*), an 'intermediate

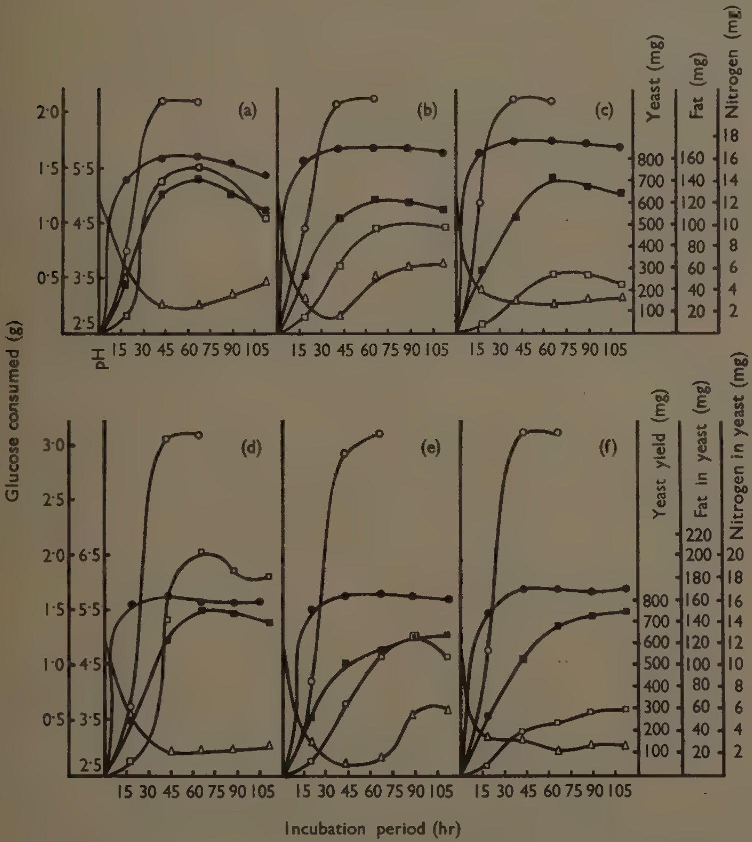

Fig. 1. The development of *H. saturnus*, *H. anomala* Y-366 and *H. subpelliculosa* in 50 ml quantities of MYE containing urea. Initial glucose concentration: a–c, 2·12 g/50 ml, d–f, 3·09 g/50 ml. Open circles, glucose consumed; full circles, nitrogen in yeast; open squares, fat in yeast; full squares, yeast yield; open triangles, pH. (a) and (d), *H. saturnus*; (b) and (e), *H. anomala* Y-366; (c) and (f), *H. subpelliculosa*.

fat' yeast (*H. anomala* Y-366) and a 'low fat' yeast (*H. subpelliculosa*) have been expressed graphically; the results for *H. ciferrii*, *H. angusta* and *Rh. gracilis* are shown in Table 4.

All the yeasts exhibited the initial phase of nitrogen uptake typical of the growth of a yeast in a 'fat' medium (Enebo, Anderson & Lundin, 1946). The bulk of the yeast mass was formed during the second phase when little nitrogen was taken up from the medium, and it is in this phase that the contrasts in fat synthesis are shown. Because the total yields of yeast in the medium containing the higher concentration of glucose are not appreciably greater than those in the medium containing the lower concentration of glucose, it appears that the synthetic capabilities of cells are limited. With the exception of *Rh. gracilis*, yeast growth continued after the glucose had been completely decomposed, which suggests that cellular synthesis was taking place at the expense of intermediates formed during glucose breakdown. One possible intermediate is ethyl acetate, which is known to be produced by *Hansenula* spp. and it was observed that whereas a fruity odour was quite pronounced at the first and second harvestings it later disappeared. Moreover, further work with *H. saturnus* and *H. subpelliculosa* has shown that most of the yeast mass is formed after the glucose has been metabolized and that in the 'low fat' *H. subpelliculosa* polysaccharide synthesis predominates during this second phase of assimilation (Hopton & Fahmy, unpublished data).

Table 4. *Data* relating to the development of selected yeasts of different types in two MYE media† with nitrogen as urea*

Yeast species and type	Period of growth (hr)	Glucose (g consumed)		Yeast (mg)		Yield (%)§		Final pH		Nitrogen in yeast (mg)		Fat in yeast (mg)	
		(a)†	(b)†	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
H. ciferrii high fat	14	1·03	1·03	249	250	24	24	3·10	3·10	14·0	13·8	7	13
	38	2·11	3·05	603	594	29	19	3·20	2·90	14·5	14·9	97	103
	62	2·10	3·06	714	684	34	22	3·50	3·20	14·8	14·3	149	152
	86			629	759	30	25	3·35	3·60	13·8	14·7	108	176
	110			558	662	27	22	3·65	3·60	13·9	14·1	76	143
H. angusta low fat	41	1·36	1·30	445	423	33	33	3·60	3·70	14·3	13·8	33	31
	89	2·08	2·75	621	587	30	21	3·65	3·35	14·7	16·0	55	50
	113	2·08	3·09	632	689	30	22	3·55	2·90	15·3	15·3	53	74
	137			561	701	27	23	3·70	3·65	16·2	15·8	49	66
	161			587	717	28	23	3·75	3·60	15·8	15·9	49	61
Rh. gracilis high fat	41	1·14	1·09	460	429	40	39	4·20	4·05	15·1	15·1	134	124
	89	1·94	1·89	597	570	31	30	4·10	4·00	15·1	15·0	220	197
	113	2·08	2·19	587	619	28	28	3·40	3·40	15·4	16·1	204	228
	137		2·39	542	613	26	26	4·05	4·05	15·4	15·3	162	203
	161		2·62	514	635	25	24	4·35	3·95	15·5	14·9	122	211

* All data refer to 50 ml cultures.

† Initial glucose concentrations: (a) 2·12 g/50 ml, (b) 3·09 g/50 ml.

§ Based on glucose consumed.

The overall pattern of growth may be similar to that of *Saccharomyces cerevisiae* where, even under highly aerated conditions, the first phase of growth in a glucose medium is essentially fermentative and is followed by an oxidative phase in which intermediates are utilized (Lemoigne, Aubert & Millet, 1954; Eaton & Klein, 1957).

DISCUSSION

Under the conditions of these experiments fat accretion by the 'high fat' yeasts *H. saturnus* and *H. ciferrii* was not as great as that by the 'standard' fat yeast *Rh. gracilis*, but both their growth rate and cell yield were superior. There are definite species differences in fat synthesizing ability, a finding similar to that reported by Enebo *et al.* (1944) in their study of *Rhodotorula* spp.

The study supports the theory that abnormal fat synthesis occurs if a culture becomes deprived of factors required for normal growth when there is still a surplus of assimilable carbon in the environment. Exponential increase is characteristic of microbial growth in a non-limiting environment. Under these conditions there is extensive protein synthesis and, in a medium containing simple nitrogen compounds, intermediates in the catabolic carbon pathway are drawn off for amino acid synthesis (Roberts *et al.* 1955). It seems possible that under conditions of nitrogen deprivation, where protein synthesis is restricted, the intermediates are channelled predominantly through other pathways such as those involved in fat and polysaccharide synthesis. Further work (Hopton & Fahmy, unpublished data) has shown that there are two quite distinct phases in the growth of *H. saturnus* and *H. subpelliculosa* in a completely synthetic medium of low nitrogen content. There is an initial phase of rapid multiplication during which most of the nitrogen is assimilated, followed by a phase (in which the behaviour of the cells can be likened to that of a resting cell suspension) in which most of the non-proteinaceous accretion occurs.

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The present address of J. W. Hopton is: Department of Microbiology, The University, Birmingham, 15.

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A FLOW REGULATOR FOR AUTOMATIC pH CONTROL

BY W. C. WRIGHT

Microbiological Research Establishment, Porton, Wiltshire

SUMMARY: A device is described for improving the automatic regulation of pH of cultures of the order of 1 litre in volume by imposing delays between small successive additions of acid or alkali to allow time for mixing.

IN PRACTICE it is sometimes difficult to control automatically the pH of laboratory cultures within close limits because of a time lag between the addition of acid or alkali and the response of the sensing electrode. The lag depends on the time required for mixing and on the relative disposition of electrodes and inlet ports; it results in repeated over-correction and consequent hunting.

The regulator to be described overcomes this difficulty. When correction is required, the acid or alkali is run into the fermenter in small amounts; a short waiting period after each addition allows mixing to take place. The additions are repeated until correction is complete.

General description

The regulator is contained in a box measuring $8 \times 4 \times 4\frac{1}{4}$ in. externally. Timing is determined by resistance-capacity circuits and cold cathode trigger valves. Four manual adjustments are provided for setting the 'on' and 'off' times of both the acid and alkali valves. The regulator is inserted into the usual control system between the controller and the solenoid valves; no other connections to it are necessary. 'On' times are variable between 0·5 and 5 sec. and 'off' times between 1 and 30 sec.

Circuit operation

The regulator comprises two similar circuits (Fig. 1), one in each pair of leads from the controller to the solenoid valves.

The operation in each channel is as follows. When correction is called for, the controller supplies D.C. to points 1 and 2, and thence through contacts RL1/4 and points 3 and 4 to the solenoid valve. At the same time the capacitor C_1 begins to charge through the resistances R_1 and R_2, and when the voltage on C_1 is high enough, the valve V_1 fires and operates the relay RL1; RL1/1 and RL1/2 close, discharging C_1 through R_5 and extinguishing V_1. RL1 is held on by current through R_4. The contacts RL1/4 break the circuit to the solenoid valve and instead supply current to the anode of V_2; RL1/3 opens and allows C_2 to charge through R_{11} and R_{12}. Eventually V_2 fires, operating RL2; RL2/1 breaks the circuit through RL1 momentarily, restoring the circuit to its original state. This cycle is repeated as long as the circuit is energized from the controller, i.e. until correction is complete.

The capacitor C_2 is electrolytic; its leakage current does affect the timing of the circuit, but it is stable enough for the present purpose.

The dials for the timing controls R_1 and R_{11} are scaled 0–10 in arbitrary units.

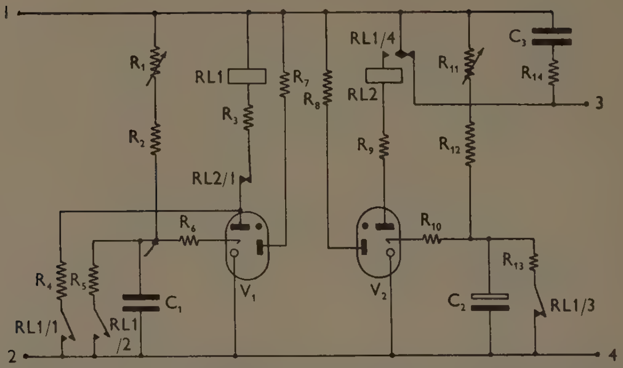

Fig. 1. Diagrammatic representation of the regulator circuit (one channel only). The values of the components are: R_1, R_{11}, 2·5 MΩ, variable; R_2, R_9, R_{12}, 56 kΩ, ½ W; R_3, 4·7 kΩ, 1 W; R_4, 15 kΩ, 1 W; R_5, R_{13}, 470 Ω, ½ W; R_6, R_{10}, 100 kΩ, ½ W; R_7, R_8, 10 MΩ, ½ W; C_1, 2μF, 200 V, paper; C_2, 8μF, 450 V, electrolytic; C_3, R_{14}, suppressor, values depend on solenoid used; RL1, 7500 Ω coil, 2 normally open, 1 normally closed, 1 change over; RL2, 3100 Ω coil, 1 normally closed; V_1, V_2, Mullard Z803U (CV2434).
The relays are shown in the unoperated condition. The terminals 1 and 2 are connected to the controller and terminals 3 and 4 to the solenoid valve.

Performance

The regulator has now been in use for 6 months with continuous cultures of volume 0·5 and 2 l.; a marked improvement in control has been obtained (Fig. 2). In addition, it has made possible the use of much stronger solutions of acid and alkali, thereby reducing volume changes in the culture.

If greater accuracy in timing were required, C_2 could be replaced by a 2μF paper capacitor and R_1 and R_{11} each by a series of fixed resistors selected by switches.

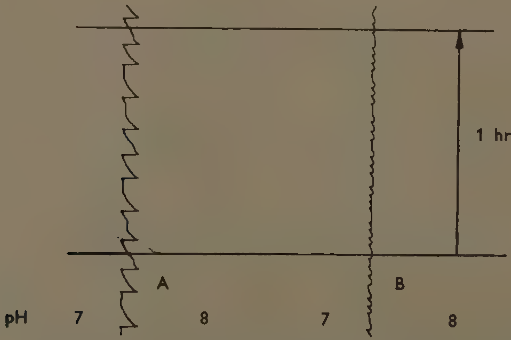

Fig. 2. Records showing the pH fluctuations in continuous cultures in which control was effected by the addition of alkali: A, without regulator; B, with regulator. Photographed from the actual charts supplied by Dr. S. J. Pirt.

The apparatus for which this circuit was designed (see Callow & Pirt, 1956) incorporated D.C. solenoids fed from a D.C. mains supply. With the addition of a rectifier and filter in the leads to the regular circuits and some modification of the RL1 contacts (separate make and break instead of RL1/4), it could be used equally well with A.C. solenoids.

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THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE VIABILITY AND GROWTH OF BACTERIA DAMAGED BY PHENOLS

BY S. E. JACOBS AND N. D. HARRIS

Bacteriology Laboratories, Imperial College of Science and Technology, London, S.W.7

SUMMARY: The effects of various environmental conditions on the viability and growth of normal *Escherichia coli* and *Staphylococcus aureus* cells, and of cells of these species which had been damaged by exposure to phenol, *o*-cresol and *p*-chloro-*m*-cresol, have been examined.

With normal cells none of the conditions tried had any effect on the initial counts, but sometimes, more often with *Staph. aureus*, the bulk of growth eventually obtained in broth media was larger, especially when the medium had been made with pyridine extracted peptone, or when it had been treated with 0·1% of Norit charcoal or 0·02% of $FeCl_3$.

In contrast, damaged cells of both species were very sensitive to their environment. At room temperature, 0·1% of Norit added to tap water adsorbed more than 80% of the cells and the initial count was increased. Nevertheless the cells died thereafter. Damaged cells inoculated into broth also died, but when Norit was present both the rate and the extent of death were markedly reduced, though few cells were adsorbed. The addition of $FeCl_3$ (0·02 or 0·03%) had no effect. At 37° damaged cells died faster than at room temperature, but the protective effect of Norit was still evident. In nephelometric studies of cultures growing in Norit treated broth the times to turbidity were shorter than those of control cultures in untreated broth, but broth treated with $FeCl_3$ often gave longer times than the controls.

In a comparison of broths prepared with peptone and/or Lab-Lemco which had been extracted with pyridine to remove suspected toxic substances, the initial counts of treated cells were highest when only the peptone had been extracted. With media containing extracted Lab-Lemco, or with both ingredients extracted, the counts were smaller but were still generally higher than in normal broth. The results of nephelometric studies were consonant with these findings: broths prepared with extracted ingredients generally gave shorter turbidity times than normal broth, especially with *E. coli*.

The addition to broth of cations, especially Mg^{2+} (10^{-3} M), favoured the survival of damaged *E. coli* but did not influence that of *Staph. aureus*. But with both species, five times that concentration of Mg^{2+} was as effective as 0·1% of Norit, which was shown to adsorb lead, tin, copper, zinc, silver and cobalt from broth.

Temperatures above 37° were unfavourable to the survival of damaged cells of both species, 44° often being completely inhibitory for *Staph. aureus*, but this effect was not due to traces of phenol in the inoculum. At 25° damaged *E. coli* recovered less well than at 30° and 37°, but damaged *Staph. aureus* did better at 25° and 30° than at 37°.

THE PROBLEM arises in several fields of applied bacteriology of obtaining growth from bacteria which have been damaged by exposure to uncongenial environments, and there is an increasing awareness that the quality of the growth medium may affect the outcome considerably. In their studies of antiseptic activity Flett *et al.* (1945) included activated charcoal or ferric chloride in the subculture medium and obtained increases in the extinction times of *Staphylococcus aureus* and *Eberthella typhosa* exposed to phenol. However, Tilley (1945) failed to confirm this. A possible reason for this disagreement was indicated by Jacobs & Harris (1953, 1954), who showed in extensive trials that presumably identical batches of nutrient broth might differ considerably in the extent to which they were improved by the addition of ferric chloride or activated charcoal (Norit). Jacobs & Harris (1954) found that the improvement did not result solely from neutralization or adsorption of phenol carried over with the inoculum. However, the possibility was not excluded that the removal of phenol carried on or in the cells produced the effects, and it was decided in further work to examine the effect of absorbents on the survival in tap water of bacteria which had been exposed to various phenolic bactericides. Tap water was chosen because it had been found (Jacobs & Harris, 1954) that Norit would adsorb about 80% of the phenol from a dilute aqueous solution, so bactericide carried on or in the cells might well be removed under these conditions. Studies with adsorbents in nutrient broth were also included so that the situation in broth, where there was little adsorption of phenol, could be compared with that in water, where adsorption was considerable. Also it was desired to see whether the agents would adsorb cells; if the majority of cells were adsorbed then this intimate contact might result in effective desorption of bactericide on or in the cells. The authors wished also to confirm the results they had reported briefly (Jacobs & Harris, 1953), that while cells damaged by phenolic disinfectants continued to decrease in numbers after being transferred to nutrient broth there was no such decrease if the broth contained Norit. These findings had suggested that normal nutrient broth contained substances which were inimical to the survival (or revival) of such damaged cells, though not to normal cells. In this connection Allen and his co-workers (Allen, Pasley & Pierce, 1952; Allen, Pierce & Smith, 1953; Childs & Allen, 1953) obtained higher counts of *Escherichia coli* and *Streptococcus faecalis* when cells from polluted water were resuscitated in nutrient broth prior to transfer to inhibitory selective media. These workers also showed that counts on bile salt media from suspensions of *E. coli* in quarter-strength Ringer's solution or phosphate buffer decreased progressively with time of contact, and suggested that these attenuated cells were more susceptible than normal ones to the inhibitory effect of certain media. Schuhardt, Rode, Foster & Oglesby (1949) have already shown that pyridine extraction will remove from peptone substances which prevent the growth of *Brucella* spp., and accordingly it was decided to study the possibility of improving nutrient broth for the revival and growth of damaged bacteria, by using in its preparation ingredients extracted with pyridine. Further, since the assessment of the viable cells in an inoculum is done normally by means of colony counts, the growth from an inoculum of damaged bacteria was examined on the modified nutrient media solidified with agar.

MATERIALS AND METHODS

Organisms and suspensions. Two organisms were used. One was the strain of *Escherichia coli* previously used by Jacobs & Harris (1953, 1954) and then referred to as *Bacterium coli* strain 2, and the other was *Staphylococcus aureus* NCTC 4736. They were grown for 24 hr on nutrient agar slopes at 37° and suspensions of washed cells in phosphate buffer of pH 7·0, containing *c.* 10^6 cells/ml, were prepared as described by Jacobs & Harris (1954). For some experiments, similar suspensions were made in glass distilled water.

Treatment of organisms with bactericides. Three bactericides were used: phenol, of Analytical Reagent quality, and *o*-cresol and *p*-chloro-*m*-cresol (PCMC), both complying with the requirements of the British Pharmacopœia (1948). The bactericide solutions were usually made in 0·02 M phosphate buffer of pH 7·0, though occasionally glass distilled water was used, and were such that the final concentrations in the reaction mixtures were: phenol, 1·0%; *o*-cresol, 0·4%; PCMC, 0·075% (all w/v). The procedure was similar to that described by Jacobs & Harris (1954), 1 ml of standard suspension being added to 4 ml of bactericide solution at 20 ± 0·5° and shaken well. The reaction was stopped when desired by diluting a sample considerably (at least 200 times) in sterile tap water or other fluid. The survivors were then counted. The combinations of treatment times and dilutions used were selected on the basis of prior trials and permitted the estimation of survivors either directly or on one further dilution at most: the ranges covered are given in Table 1.

Table 1. *The ranges of exposure time, and subsequent dilution to stop the reaction, which gave numbers of bactericide treated cells suitable for colony counts*

Solvent	Bactericide	*E. coli*		*Staph. aureus*	
		Times (sec)	Dilutions	Times (sec)	Dilutions
PO$_4$ buffer, 0·02 M, pH 7·0	Phenol	35–120	1:200 to 1:4000	30–45	1:200 to 1:2000
	o-Cresol	35–90		25–55	
	PCMC*	50–100		35–75	
Glass distilled water	Phenol	285–450	1:20,000 to 1:200,000	60–105	1:4000
	o-Cresol	375–420		90	
	PCMC*	390–405		105	

* PCMC, *p*-chloro-*m*-cresol.

Colony counts. These were done by the surface viable method of Miles & Misra (1938), with 5 replicate 0·02 ml drops at each dilution, using nutrient agar of the composition described below. When dilution was needed before plating it was usually done in sterile tap water but if broth was used the colony counts were multiplied by 1·1 to allow for the smaller drop size. Colonies were counted using a hand lens after 48 or 24 hr incubation at 37°. The 48 hr count was used whenever possible since experience showed that the numbers were then maximal, but sometimes with high colony densities (⩾50/drop) the growth was confluent at 48 hr and the 24 hr count had to be used. Some overcrowding effects may have occurred in such cases, and with other less extreme instances of high colony density. On other occasions low counts of 5/drop or less were the only ones available, and here sampling errors may have been large. Counts which were unsatisfactory for these reasons did not, however, upset the general trends of the results, so they have been included, especially since complementary information obtained by the use of other techniques confirmed those trends. For clarity in presentation only the mean values have been given in the Tables.

Determination of growth curves. The course of growth of cultures inoculated from suspensions which had been exposed to the different bactericides was determined in various media. The rapid and convenient nephelometric method was used and the instrument (Evans Electroselenium Ltd., Harlow, Essex) was calibrated in terms of viable cells (determined by surface viable counts on nutrient agar). Full details of the calibration procedure have been given by Harris (1953). That satisfactory results were obtained was indicated by the coefficients of variation of the estimates of viable cells/unit increase in turbidity, which were 6·33 for *E. coli* and 20·1 for *Staph. aureus*.

The media to be tested were distributed aseptically in 5 ml portions into matched $6 \times \frac{5}{8}$ in. Pyrex test tubes. After sterility had been proved by incubation, a tube of each medium was inoculated (at random) with 0·02 ml of a suitable dilution of a suspension of unexposed or bactericide exposed cells and at the same time a colony count was performed to determine the number of viable cells inoculated. The inoculated tubes were then held at 20° for 24 hr, after which they were transferred to a waterbath at 37°. This procedure was adopted because continuous incubation at 37° led, with the low inoculation levels employed, to the development of turbidity at most inconvenient times during the night. Continuous incubation at a lower temperature was considered undesirable on the ground that the temperature of incubation ought not to be greatly different from that prevailing in the colony count method. The use of larger inocula might have brought the development of turbidity within the span of a working day, but it was especially desired to use small inocula of the order of 10/ml and, by using two temperatures, detectable turbidity and rapid growth occurred on the day following inoculation.

After transfer to 37°, turbidity readings were taken at 30 min intervals for 10 hr, and again at 24 hr and 48 hr, and the growth curves plotted.

Media, and pyridine extraction of ingredients. The nutrient broth employed contained: peptone (Oxoid no. L 37), 10 g; Lab-Lemco, 10 g; sodium chloride, 5 g; tap water, 1 l. Nutrient agar was prepared from this medium and contained 15 g/l. of Oxoid New Zealand agar.

In the preparation of modified media either or both the peptone and Lab-Lemco had been extracted with pyridine, and these were used at the above concentrations. Initially, samples of peptone and Lab-Lemco were dried at 100° for 3 days and then extracted with redistilled pyridine in a Soxhlet apparatus for 8 hr at atmospheric pressure (b.p. of pyridine, 115°): but this treatment proved rather drastic and in later work the broth ingredients were held in contact with pyridine for 24 hr at 37°, with shaking at intervals. After decanting, the residual pyridine was removed by vacuum distillation.

All media were sterilized at 15 lb/in^2 for 20 min.

Treatment of broth with Norit and ferric chloride. In an attempt to improve the quality of nutrient broth for growing damaged bacteria, the broth was treated with either Norit or ferric chloride. Norit (0·1% w/v) or ferric chloride (0·02 or 0·03% w/v) were added to broth and, after shaking for 30 min, the mixtures were raised to boiling point. This coagulated the precipitate which formed in the broth containing ferric chloride. The broths were then filtered, distributed and sterilized. A control broth was treated similarly except that the adjuvant was not present. The addition of ferric chloride did not change the pH of the nutrient broth.

The precipitate (FeP) thus obtained from broths to which ferric chloride had been added was washed thoroughly with hot distilled water and was used in the experiments on the adsorption of cells from tap water, the results of which are reported in Table 4. In each case the FeP was prepared from broth of the same batch as was used in the rest of the experiment.

Assessment of the suitability of media for bacterial growth. To assess the suitability of a liquid medium for the growth of damaged and undamaged bacteria, three criteria were used: the time to just detectable turbidity, the growth rate in the logarithmic phase, and the maximum population, reached in the stationary phase.

The turbidity times were related to the commencement of incubation at 37° and were determined objectively by calculating the intersection of the logarithmic portion of the growth curve with a line drawn parallel to the abcissa through the logarithm of the lowest population detectable by the nephelometer under the conditions used. These populations were $5{\cdot}4 \times 10^6$ cells/ml for *E. coli* and $3{\cdot}2 \times 10^6$ cells/ml for *Staph. aureus*. The turbidity times do not represent lag phases, not only because of the preceding period of incubation at 20° but also because a period of growth was always involved, the few viable cells of the inoculum having needed to multiply considerably. Nevertheless, differences in the turbidity times obtained in the different media following inoculation of equal volumes from the same suspension of treated bacteria must reflect differences in the suitability of the media. The time differences relative to the standard nutrient broth have been taken as measures of suitability. However, a turbidity time is compounded of three elements: the effect of the medium on the ultimate viability of damaged cells, the lag period and the growth rate. These factors are not necessarily related and it was desired to avoid equating media which gave similar turbidity times but had different growth rates and different revival capacities. To guard against this, the growth rates in the logarithmic phase were determined as calculated regressions of log population on time. Also control experiments were done using undamaged cells.

For the third criterion of suitability, the maximum population in the stationary phase was recorded 24 or 48 hr after the start of incubation at 37°.

The suitability of a solid medium was judged by the colony count which it yielded.

EXPERIMENTS AND RESULTS

The effects of Norit and ferric chloride broth precipitate (FeP) in tap water, and Norit and ferric chloride in nutrient broth, on the survival in these fluids of unexposed and bactericide exposed bacteria

For each trial six fluids were prepared, three based on tap water and three on nutrient broth. Two of the fluids were unmodified tap water and broth, two were those fluids plus Norit and the others were tap water plus FeP and nutrient broth plus ferric chloride. The amounts of these were such that in the final mixtures of 45 ml of fluid plus 5 ml of bacterial suspension the Norit concentration was $0{\cdot}1\%$ (w/v) and the iron concentration was equivalent to that in $0{\cdot}03$ or $0{\cdot}02\%$ (w/v) $FeCl_3$.

To 45 ml portions of these fluids sterilized in screw capped bottles were added 5 ml portions of appropriate dilutions in sterile tap water of a suspension of unexposed or bactericide exposed *E. coli* or *Staph. aureus*. The final dilution of the suspension was 1:200, i.e. approximately that used in the extinction time experiments described by Jacobs & Harris (1954). The bottles were then shaken vigorously and samples plated immediately on nutrient agar to obtain the initial count. The bottles were then left at room temperature (18–20°) for 3 hr, during which time the adsorbents settled out. Samples of the supernatants, and of the whole fluid after shaking to resuspend the adsorbents, were then plated. This process was repeated after the fluids had stood at room temperature for a further 3 hr, and yet again after a further 18 hr, so that counts were done after 0, 3, 6 and 24 hr standing. Since only small volumes were withdrawn on each occasion of sampling the supernatant, the errors introduced thereby were negligible. Also, it was assumed that slow death was occurring in the initial dilution of an exposed cell suspension, so that the fluid inoculated last from such a dilution, some 2 min after the first, would have received a smaller initial inoculum. Accordingly, after the preliminary experiments had indicated the fluids which most favoured survival, those fluids were always inoculated last to avoid exaggerating any initial favourable effect.

Table 2. *The effect of Norit at room temperature on the survival in tap water and on the survival and growth in broth of normal and bactericide treated cells of* E. coli *and* Staph. aureus

Time of contact with fluid (hr)	Suspension fluid	Mean relative viable count* of normal and bactericide treated							
		E. coli				*Staph. aureus*			
		Normal	Phenol	*o*-Cresol	PCMC	Normal	Phenol	*o*-Cresol	PCMC
0	Tap water	100	100	100	100	100	100	100	100
	Tap water + Norit	106	200	345	184	94	363	276	198
	Broth	100	135	130	99	96	112	126	101
	Broth + Norit	102	135	123	110	91	189	221	127
3	Tap water	104	8	19	9	86	2	0	0
	Tap water + Norit	105	89	221	121	85	26	40	31
	Broth	113	41	91	46	100	50	58	49
	Broth + Norit	108	132	156	154	97	281	175	107
6	Tap water	106	4	8	2	75	0	0	0
	Tap water + Norit	106	58	187	46	89	8	22	10
	Broth	286	26	66	13	134	27	52	28
	Broth + Norit	334	114	153	80	151	155	186	76
	Number of trials	4	5	2	2	4	6	2	2

* On nutrient agar, relative to counts from tap water suspensions at 0 hr. The counts were done on the whole fluids, including Norit where this was present.

With each organism four complete replicate trials were carried out using unexposed cells and two more using cells treated with each bactericide. The mass of data thus obtained is too large for full presentation, but part of it is summarized in Table 2. This gives the mean values for the counts on the whole fluids without and with Norit up to 6 hr, relative to the mean initial count in tap water taken as 100 to facilitate comparison. The use of mean values was considered justifiable, because the responses in the various fluids were always of the same kind in the various trials: but it should be realized that the magnitude of the response differed in the individual trials, especially after 24 hr, probably because differences in laboratory temperature affected both the duration of the lag phase and the rate of growth in the logarithmic phase.

Unexposed cells. With *E. coli* in tap water there was no change of note in 6 hr and only a small increase after 24 hr. This was also the case when Norit was present, but with FeP in tap water there was a steady small increase during 6 hr and a marked rise after 24 hr, indicating that nutrients were present, either adsorbed on the FeP or combined with the iron. In the broth media without adsorbents the slight growth which occurred in all cases after 6 hr became considerable after 24 hr. The amount of growth was not markedly affected by the adsorbents, though they seemed to increase it somewhat.

In contrast, unexposed *Staph. aureus* died slowly in tap water. This was possibly retarded a little by Norit, but FeP prevented it and after 24 hr there was some growth, though much less than with *E. coli*.

Bactericide treated cells. Both *E. coli* and *Staph. aureus* died in tap water, the latter more rapidly than the former. Death occurred also during the first 6 hr in broth, but it was slower than in tap water and in fact *E. coli* exposed to *o*-cresol or PCMC, and *Staph. aureus* exposed to *o*-cresol, made notable growth after 24 hr.

The effect of Norit in tap water was to raise the initial count of both organisms; but after this death occurred. Norit in broth, in contrast, did not raise the initial count of *E. coli* and raised that of *Staph. aureus* to a comparatively small extent, but it had a striking effect in reducing the rate and extent of the continuing death to which both organisms were subject in plain broth, and led to larger counts after 24 hr.

The addition of FeP to tap water caused some enhancement of the initial count of *Staph. aureus*, though not of *E. coli*, and it also led to a reduction in the death rate which occurred in its absence. In this it seemed somewhat less effective than Norit. When present in broth, ferric chloride exerted no favourable effect on survival.

Further limited experiments were then done on the survival at 37° of phenol exposed organisms in broth with and without 0·1% (w/v) of Norit, to compare with the results obtained at room temperature. The results are shown in Table 3, and

Table 3. *The effect at* 37° *of Norit* (0·1% *w/v*) *on the survival of phenol treated bacteria in nutrient broth*

Time of contact with fluid (hr)	Suspension fluid	Mean viable count* of			
		E. coli		*Staph. aureus*	
		Trial 1	Trial 2	Trial 1	Trial 2
0	Broth	>250	111	37	3·0
	Broth + Norit	>250	c. 250	126	21·6
1	Broth	70	6	4	0·4
	Broth + Norit	>250	c. 250	103	25·6
2	Broth	47	3	3	0·2
	Broth + Norit	>250	>250	91	26·6
3	Broth	75	6	3	0·2
	Broth + Norit	>250	>250	93	36·2
4	Broth	>250	22	3	0·4
	Broth + Norit	>250	>250	97	37·2

* Cells/0·02 ml drop (mean of 5 drops) on nutrient agar. The counts were done on the whole fluid, including Norit where this was present.

clearly there was continuing death at the higher temperature also. Comparison with Table 2 shows that it was faster than at room temperature. The protective effect of Norit in the broth was also evident at 37°, and it was perhaps more marked than at the lower temperature.

Table 4. *The adsorption by Norit and FeP of normal and bactericide treated organisms from tap water and nutrient broth*

Organism and time of contact with fluid (hr)		Suspension fluid	Adsorbent	Mean percentage of organisms adsorbed, with cells				
				Untreated	Phenol* treated	Phenol† treated	*o*-Cresol† treated	PCMC† treated
E. coli	3	Water	Norit	93	87	86	81	81
			FeP	87	83	23	33	9
		Broth	Norit	2	4	—	2	9
			$FeCl_3$	2	0	—	2	18
	6	Water	Norit	96	95	97	86	95
			FeP	93	92	16	31	51
		Broth	Norit	3	—	3	—	12
			$FeCl_3$	1	28	28	16	32
Staph. aureus	3	Water	Norit	87	89	96	94	87
			FeP	92	87	57	59	60
		Broth	Norit	25	18	28	25	29
			$FeCl_3$	43	70	76	73	63
	6	Water	Norit	90	92	96	97	93
			FeP	92	87	55	91	62
		Broth	Norit	10	8	16	15	7
			$FeCl_3$	52	28	39	32	42

* Here 0·03% (w/v) $FeCl_3$, or its equivalent as FeP, were used. † Here 0·02% (w/v) $FeCl_3$, or its equivalent as FeP, were used.

The adsorption of bacteria by Norit and FeP. The extent to which *E. coli* and *Staph. aureus* cells were adsorbed on Norit and FeP can be calculated from the proportions of the counts in the supernatants to those in the whole fluids, i.e. the proportion of unadsorbed cells. The mean values from the experiments done at room temperature are shown in Table 4. Both *E. coli* and *Staph. aureus* were strongly adsorbed from water by Norit, which removed 81–97% of the cells. FeP also adsorbed bacteria from water, but the effect was generally smaller and less consistent than with Norit and depended markedly on the amount of FeP present. There was much less adsorption of cells from broth than from water: Norit did not adsorb *E. coli* and adsorbed *Staph. aureus* only slightly; but FeP was more active, especially with *Staph. aureus*.

The effect of treating broth with Norit or ferric chloride on its suitability for the growth of normal and damaged bacteria

Growth curves were determined nephelometrically in nutrient broth, untreated and treated with Norit or ferric chloride as described above, which had been inoculated with normal *E. coli* or *Staph. aureus* cells or with cells from suspensions exposed to the bactericides. Two batches of broth were used: the first was treated with 0·03%

ferric chloride but, since this treatment proved harmful to the recovery of treated cells, 0·02% was used with the second batch. Many trials were made, because it was difficult with treated organisms to obtain samples for inoculation which contained sufficient but not too many cells capable of growth in all the different media. With too few cells, growth did not occur at all in the untreated broth so that despite the obvious disparity between the media an exact numerical value for the difference between the turbidity times could not be recorded, while with too large an inoculum turbidity appeared during the period of incubation at 20° and no result was obtained. In all cases the number of cells in the inoculum was estimated by means of a surface viable count on the standard nutrient agar.

Growth in the logarithmic phase. The regressions of log population on time were calculated for each trial, and the regression coefficients are given in Table 5. It is evident from the generally large ratios of the coefficients to their standard errors that the data are adequately represented by the linear regressions and that true logarithmic growth was occurring. The upper limits for this phase were *c.* 300×10^6 cells/ml for *E. coli* and *c.* 35×10^6 cells/ml for *Staph. aureus*.

Table 5. *The occurrence of true logarithmic growth from inocula of untreated and bactericide treated cells in cultures growing in normal and modified broth media*

Treatment of bacteria	Treatment of broth	Growth rates (M)* with their standard errors (S.E.)† and ratios					
		E. coli			*Staph. aureus*		
		M	S.E.	M/S.E.	M	S.E.	M/S.E.
None	None	7648	1609	4·8	4514	387	11·7
	$FeCl_3$	6510	1198	5·4	4311	405	10·7
	Norit	8770	936	9·4	4804	247	19·4
Phenol	None	7333	106	6·9	2597	170	15·3
	$FeCl_3$	9225	467	19·8	4005	519	7·7
	Norit	6873	426	16·1	3970	765	5·2
o-Cresol	None	7049	447	15·8	6063	988	6·1
	$FeCl_3$	9805	2371	4·1	4139	457	9·1
	Norit	8852	1072	8·3	6063	1001	6·1
PCMC	None	8657	337	25·7	4418	1311	3·4
	$FeCl_3$	6765	336	20·2	5480	1136	4·8
	Norit	8115	559	14·5	4005	518	7·7

* Calculated coefficients of regression of $\log_{10}$ (cells/ml) on time (hr), $\times 10^4$. † Standard errors, $\times 10^4$.

Although the data were extensive, the failure of some cultures to show a turbidity within the observation period resulted in missing values, so that the results obtained with one batch of medium were insufficient for a rigorous statistical analysis. An analysis of variance of the data for the other batch indicated that treatment of the medium may have influenced the growth rate of *E. coli* (*P*, *c.* 0·035) but the growth rates of *Staph. aureus* in the various media were not significantly different. However, in view of the variability of the data, it was felt that more information was needed to decide this point and, to avoid bias, the turbidity times were computed from the individual calculated slopes in each trial.

Table 6. *The effect of Norit* (N) *and ferric chloride* (F) *treatments of nutrient broth on the turbidity times* (t, hr) *for cultures inoculated with untreated and bactericide treated organisms*

Organism, medium batch and trial no.			Inoculum (I)* and decreases (ΔtN, ΔtF) in t, with cells											
			Untreated			Phenol treated			*o*-Cresol treated			PCMC treated		
			I	ΔtN†	ΔtF‡	I	ΔtN†	ΔtF‡	I	ΔtN†	ΔtF‡	I	ΔtN†	ΔtF‡
E. coli	36§	1	—	—	—	1·8	>15·9	—	2·0	>15·8	—	44·0	0·0	−0·6
		2	—	—	—	2·4	4·1	−>14·9	2·4	>17·5	—	0·4	14·0	—
		3	—	—	—	16·0	>3·3	>0·5	5·4	>3·3	—	1·4	14·0	—
	37**	1	2·6	0·3	0·2	4·4	>1·5	—	28·0	0·5	−0·5	24·6	1·6	0·4
		2	4·0	−0·1	−0·9	12·0	2·1	−0·5	21·0	0·7	−>5·9	30·0	0·9	−2·1
		3	1·8	0·6	0·5	50·0	0·0	0·0	38·0	−0·3	0·0	42·0	−0·3	−0·7
Staph. aureus	36§	1	—	—	—	76·0	−0·1	−2·7	3·4	3·9	−>14·0	50·0	1·6	−1·5
		2	—	—	—	42·0	2·8	−2·8	3·0	>16·0	—	26·2	1·3	−1·7
		3	—	—	—	0·4	1·6	−>16·5	1·0	3·1	−>1·7	1·0	2·3	−0·5
	37**	1	2·2	0·5	0·0	10·0	1·9	−>4·7	20·0	1·7	−0·6	30·0	2·3	−2·3
		2	4·4	0·6	−0·4	7·2	0·0	−3·0	29·2	1·0	0·0	29·6	0·4	−1·8
		3	4·6	0·1	0·2	98·0	2·6	−2·4	42·2	2·2	−1·7	36·4	2·5	−1·5

* Cells/ml, counted on nutrient agar. § Treated with 0·03% $FeCl_3$. ** Treated with 0·02% $FeCl_3$.
† The symbol '>' under ΔtN indicates that there was no growth at 10 hr, and sometimes not at 24 hr, in the control broth (no Norit). ‡The symbols '−>' under ΔtF indicate that there was no growth at 10 hr, and sometimes not at 24 hr, in the broth treated with $FeCl_3$.

Turbidity times. Since the growth rates in the different media of the various kinds of cells did not differ greatly, the turbidity times would have been affected similarly by that part of the logarithmic growth phase included therein. Differences in turbidity times could thus be regarded with confidence as measures of the relative suitabilities of the media for the survival of the inoculated cells.

The effects of Norit and ferric chloride treatment of nutrient broth are shown in Table 6, in terms of the differences between the turbidity times of the cultures in the normal and treated broths. There were three trials in each of two batches of broth with each organism, and there was a direct comparison between the untreated and treated broths because equal inocula from the same sample of cells were put into all three broths.

Dealing with the effect of Norit treatment first, with untreated cells of both organisms there was a tendency for the turbidity time to be slightly shorter, but with treated cell inocula there were often marked reductions. It should be noted that where in Table 6 the symbol '>' appears against a ΔtN value, the cells inoculated into the untreated broth had failed to develop sufficiently within the 10 hr period during which regular half-hourly observations were made, or even on occasion by the end of the experiment, i.e. 24 or 48 hr after incubation at 37° had been begun. It is also noteworthy that there was a tendency, especially with *E. coli*, for the greatest effects to be associated with the smaller inocula.

As regards the effect of ferric chloride treatment, most of the ΔtF values are negative, which means that the turbidity times for the treated broths were longer than those for untreated broth. Here, the '>' sign means that the cells inoculated into the treated broths did not grow within the experimental period. It is therefore clear that ferric chloride treatment rendered the broth very much less suitable for the revival of damaged bacteria.

Population densities. The relative population densities reached after 24 hr, expressed as a percentage of those attained in untreated broth, are given in Table 7.

Similar effects were observed whether the cells of the inocula had or had not been exposed to a bactericide. In the case of *Staph. aureus* the populations were markedly higher when the broth had been treated with ferric chloride, and there was a similar but smaller response in Norit treated broth. In contrast, *E. coli* grew only slightly more abundantly in ferric chloride treated broth, and rather less well in Norit treated broth, than in the control. The differences were statistically significant only in the experiments where *Staph. aureus* had been exposed to *o*-cresol, since the population density was generally rather variable; but the improved growth of *Staph. aureus* when media were treated with ferric chloride or (to a lesser extent) with Norit, was confirmed by observations of colonies on solid media: these were somewhat larger as a result of Norit treatment of the broth and considerably larger when the ferric chloride treatment had been given.

Table 7. *The effect of Norit and ferric chloride treatment of nutrient broth on the populations after 24 hr at 37° in cultures inoculated with untreated and bactericide treated organisms*

Organism and broth treatment		Mean percentage populations (M)* and numbers of replicates (R) with							
		Normal cells		Cells exposed to					
				Phenol		*o*-Cresol		PCMC	
		M	R	M	R	M	R	M	R
E. coli	Norit	98	3	113	5	108	4	98	4
	$FeCl_3$	110	3	132	3	99	3	106	4
Staph. aureus	Norit	129	3	132	6	130	5	138	6
	$FeCl_3$	216	3	171	5	229	4	220	6

* Relative to the untreated broth value taken as 100, which represented *c.* 1200×10^6 *E. coli*/ml and *c.* 300×10^6 *Staph. aureus*/ml.

Experiments with other adsorbents

From time to time suggestions have been made that nutrient media might be improved in various ways if inhibitory substances thought to be present therein could be removed. Unspecified colloidal materials were supposed by Wyss (1948) to act in this way. The use of serum was advocated by Süpfle & Dengler (1916) and by Flesch (1921), and serum albumen by Pollock (1947), while soluble starch was employed in the work of Olsen & Scott (1950) and Murrell, Olsen & Scott (1950). Trials were made therefore to discover whether the inclusion of horse serum, egg albumen and gum acacia (all at 1·0 and 0·3% w/v), and soluble starch (at 0·1% w/v) would improve nutrient broth for the growth of normal or bactericide exposed *E. coli* and *Staph. aureus*, but the results were irregular and no consistently favourable effects were observed either in nephelometric growth curve experiments or in extinction time studies. Indeed, the growth curve data showed that horse serum hindered the growth of cultures inoculated with damaged cells.

The inclusion of soluble starch in solid media also had no apparent effect.

The suitability for the growth of normal and damaged bacteria of media prepared with pyridine extracted ingredients

Experiments with solid media

In preliminary experiments the pyridine extraction of peptone and meat extract was done at 115°, and not surprisingly the growth capacities of the modified media, as judged by the numbers of colonies obtained from a fixed inoculum, were impaired. But nevertheless, on a medium in which both the peptone and Lab-Lemco had been extracted with pyridine, phenol treated bacteria of both species showed higher counts than on the control medium on two out of three occasions of testing. Counts of unexposed bacteria were not enhanced, and were even slightly lower on some of the media.

Further experiments were then done using media prepared with ingredients which had been extracted at 37°, and the results of one trial are presented in Table 8.

Table 8. *The effect of using pyridine extracted peptone and Lab-Lemco in agar media on the apparent viability of untreated and bactericide treated organisms*

Organism	Ingredient(s) extracted at 37° with pyridine	Mean colony count/drop*				Mean overall response of treated cells†
		Normal cells	Cells exposed to			
			Phenol	*o*-Cresol	PCMC	
E. coli	Neither	20·2	4·6	2·2	2·6	1·0
	Lab-Lemco	20·8	56·0	17·8	11·2	5·3
	Peptone	22·0	67·0	23·2	35·0	14·5
	Both	22·0	11·4	5·4	2·4	1·8
Staph. aureus	Neither	17·2	50·0	25·6	27·2	1·0
	Lab-Lemco	15·4	73·6	110·4	57·2	1·9
	Peptone	18·6	153·0	172·6	149·6	4·8
	Both	15·2	74·6	9·8	53·6	1·8

* Detailed results of one trial. † Mean of 12 trials (4 with each bactericide), relative to count on normal nutrient agar as unity.

In all, with each organism two trials were done with untreated bacteria and four with cells treated with each bactericide, and the data presented in Table 8 are typical. There were no appreciable differences in the counts of untreated bacteria on all four media. Counts on media prepared with extracted peptone and normal Lab-Lemco invariably gave greatly enhanced counts of treated organisms, the response being greater with *E. coli* than with *Staph. aureus*. With media prepared from extracted Lab-Lemco and normal peptone the counts were usually (10 times out of 12) higher on the control medium with treated *E. coli*; but with treated *Staph. aureus*, and with both organisms on the medium with both ingredients extracted, the counts were greater and less than those on the control medium with approximately equal frequency. However, as shown in the last column of Table 8, there was on the whole an increase in count on all the media with one or both ingredients extracted, with both organisms.

Experiments with liquid media

Growth curves were determined nephelometrically in media prepared from ingredients extracted with pyridine at 37° and inoculated from normal and bactericide treated suspensions of *E. coli* and *Staph. aureus*. The numbers of cells inoculated were estimated by making surface viable counts on the standard nutrient agar.

Growth in the logarithmic phase. In these experiments the growth rates were calculated as the regressions of log population on time. As with the Norit and ferric chloride treatments the data were not sufficiently complete to permit of thorough statistical analysis, but it seemed probable that the growth rates in the various media were different: more extensive experiments are needed to decide this point conclusively. Accordingly turbidity times were calculated as described for the Norit and ferric chloride treatments.

Turbidity times. The differences between the times taken in the control and modified media with one or both ingredients pyridine extracted are shown in Table 9. The use of extracted ingredients clearly improved the suitability of the media for damaged *E. coli*, but with damaged *Staph. aureus* the improvement, though consistent, was relatively small. As in the experiments with Norit treated media the control cultures were sometimes sterile although growth occurred in the modified media. With undamaged cells the differences were small and rather variable.

Table 9. *The effect of using pyridine extracted peptone and Lab-Lemco in broth on the turbidity times of cultures inoculated with untreated and bactericide treated organisms*

Organism and trial no.		Ingredient(s) extracted at 37° with pyridine	Inoculum (I)* and decreases in turbidity times (Δt, hr)†§ with							
			Untreated cells		Cells treated with					
					Phenol		*o*-Cresol		PCMC	
			I	Δt	I	Δt	I	Δt	I	Δt
E. coli	1	Lab-Lemco	3·6	−0·4	24·0	5·3	17·0	6·2	1·0	>45·2
		Peptone		−0·4		6·3		6·1		>44·8
		Both		−0·8		3·8		6·1		>44·6
	2	Lab-Lemco	4·0	0·7	19·2	>9·2	50·0	3·9	28·0	1·8
		Peptone		−0·2		>9·1		3·7		1·8
		Both		0·7		>9·7		4·1		2·4
	3	Lab-Lemco	—	—	1·2	7·2	22·0	>9·0	2·6	>45·5
		Peptone		—		7·3		>8·6		>45·6
		Both		—		7·3		>8·5		>45·1
Staph. aureus	1	Lab-Lemco	2·0	−0·2	0·6	>15·0	1·4	0·2	0·4	1·0
		Peptone		−0·2		>15·7		0·6		2·9
		Both		1·4		>15·0		1·2		0·9
	2	Lab-Lemco	2·4	0	11·0	2·5	22·6	0·9	28·0	0·3
		Peptone		0·2		2·1		0·9		0·2
		Both		−0·7		2·3		0·9		0·7
	3	Lab-Lemco	—	—	9·0	0·2	7·0	3·2	8·0	2·7
		Peptone		—		−0·1		2·1		1·9
		Both		—		1·0		2·5		0·1

* Cells/ml, counted on nutrient agar. † Δt, turbidity time in normal broth minus turbidity time in modified broth. § The symbol '>' indicates that there was no growth in the control broth at 10 hr, and sometimes not at 48 hr.

Table 10. *The effect of using pyridine extracted peptone and Lab-Lemco in broth media on the populations after 48 hr at 37° in cultures inoculated with normal and bactericide treated cells*

Treatment of cells of inoculum	Ingredient(s) extracted at 37° with pyridine	Population densities (10^6/ml) in trials with						Mean densities‡	
		E. coli			*Staph. aureus*			*E. coli*	*Staph. aureus*
		1	2	3	1	2	3		
None	Neither	1850*	1700*	—	312	265	—	100	100
	Lab-Lemco	1160*	1015*	—	265	186	—	61	78
	Peptone	2430*	1770*	—	596	304	—	118	153
	Both	1580*	1360*	—	257	296	—	83	97
Phenol	Neither	2960	3110	3070	—	258	209*	100	100
	Lab-Lemco	1270	1820	1700	127*	191	112*	52	64
	Peptone	3690	4130	4190	170*	265	233*	131	107
	Both	2590	3790	2960	134*	224	141*	102	87
o-Cresol	Neither	2970	2820	2970	350	240	410	100	100
	Lab-Lemco	1070	1400	1780	208	208	292	49	79
	Peptone	4370	3700	4180	500	292	573	139	138
	Both	2540	2630	3200	283	208	308	96	81
PCMC	Neither	—	3550	—	265	296	363	100†	100
	Lab-Lemco	1610	1350	1525	265	241	257	38†	84
	Peptone	4020	3920	4230	364	363	496	110†	132
	Both	2720	2300	3200	241	202	288	65†	80

* Values after 24 hr. † Results of one trial only. ‡ Relative to normal broth taken as 100.

Population densities. In Table 10 the population densities reached in trials with cultures inoculated from normal and bactericide exposed suspensions of both organisms are given, together with the mean values relative to the population densities reached in normal broth taken as 100. Evidently the growth capacities of the modified media were very different and the relative growth supporting capacities were similar no matter what treatment the cells had received prior to inoculation. As compared with normal broth, the medium with pyridine extracted peptone was notably better, that with pyridine extracted Lab-Lemco was markedly poorer, and the medium with both its ingredients extracted was generally a little less good than normal broth. These results suggest that extraction with pyridine had removed nutrients as well as growth inhibitors and that the Lab-Lemco in particular had suffered a loss of nutrients.

These results were confirmed for *Staph. aureus* by observations of the colony sizes obtained on the corresponding media solidified with agar. Observations of the colony sizes of *E. coli* on the solid media were less satisfactory, since with increased growth this organism gave more convex colonies rather than colonies of larger diameter.

The role of cations in the inhibitory properties of media

It is known that charcoal can adsorb cations (Mukherjee, 1922), and blood charcoal was used by Bortels (1927) to remove ions from media. Mulder (1948) used Norit with ammonium sulphide for the same purpose. The improvement of broth for the growth of damaged bacteria, reported above, may have been due to cation removal, especially since Maurice (unpublished data) has shown that di- and trivalent cations reduce the lethal activities of phenolic compounds. Also Massart (1952) has reviewed the evidence for competition between cations and chemotherapeutic cationic substances.

Experiments in water. Normal and damaged bacteria were diluted in glass distilled water and the required ions (as chlorides) added aseptically. As shown by viable counts, the addition of Na^+ and K^+ (10^{-2} M) led to irregular effects of no significance: but Ca^{2+} and Mg^{2+} (10^{-3} M) favoured survival, as also did Ringer's solution at one-tenth normal strength, with normal and damaged cells of both species. However, Fe^{3+} and Al^{3+} (10^{-6} M) tended to increase the death rate, despite the low concentration used.

Experiments in broth. In similar experiments in broth 10^{-2} M K^+ had little effect, but the addition of Ca^{2+} and more especially Mg^{2+}, both at 10^{-3} M, considerably favoured the survival of bactericide treated *E. coli* and Al^{3+} at 10^{-4} M had a similar action (Table 11). The addition of Fe^{3+} (10^{-4} M) gave inconsistent results with no distinct trends. The survival of *Staph. aureus* was not favoured or hindered by the addition of the above cations at these concentrations and the results are not presented.

Table 11. *The effect of cations on the survival at 20° of bactericide treated* E. coli *in nutrient broth*

Cation* added and concentration	Mean colony count† after		
	0 hr	3 hr	6 hr
None	100	20	10
K^+, 10^{-2} M	92	26	10
Ca^{2+}, 10^{-3} M	93	27	15
Mg^{2+}, 10^{-3} M	95	43	36
Al^{3+}, 10^{-4} M	105	37	15
Fe^{3+}, 10^{-4} M	97	33	13

* All as chlorides. † Mean from 2 trials with each of the 3 bactericides, expressed as a percentage of the initial count in normal broth.

Table 12. *A comparison of the effectiveness of Norit* (0·1% *w/v*) *and* $MgCl_2$ (5×10^{-2} *M*) *in promoting the survival at* 20° *of bactericide treated organisms in nutrient broth*

Time of contact (hr)	Addition to nutrient broth	Mean colony count/drop, of					
		E. coli, exposed to			*Staph. aureus*, exposed to		
		Phenol	*o*-Cresol	PCMC	Phenol	*o*-Cresol	PCMC
0	None	11·4	1·6	35·2	100·0	15·0	19·6
	Norit	12·8	2·0	33·0	83·0	15·4	20·6
	$MgCl_2$	12·6	1·6	35·8	93·6	17·2	19·6
3	None	3·6	0·8	13·0	29·2	7·8	7·2
	Norit	9·8	2·0	25·0	57·6	12·2	12·0
	$MgCl_2$	6·4	1·0	22·4	50·6	11·6	9·4
6	None	3·8	0·4	6·6	31·4	7·2	8·0
	Norit	8·0	1·2	19·0	49·2	10·6	12·4
	$MgCl_2$	8·0	1·0	21·0	41·0	10·0	10·0
24	None	3·8	0·0	8·4	49·0	4·0	7·8
	Norit	59·0	1·6	43·0	87·2	13·0	36·6
	$MgCl_2$	40·2	1·6	101·6	57·0	12·8	24·8

In further experiments it was shown that the addition of 5×10^{-3} M $MgCl_2$ to broth was quantitatively as effective as 0·1% w/v of Norit in protecting damaged cells of both *E. coli* and *Staph. aureus* (Table 12).

The removal of cations from broth by Norit. In view of the beneficial effect of adding Ca^{2+} and Mg^{2+} to broth, the improvement effected by Norit in the suitability of broth for the survival of damaged bacteria could not have been due to the removal of those ions, but it might have been due to adsorption of toxic cations such as those of heavy metals which are known to occur in commercial peptones, sometimes in considerable amounts.

Accordingly a sample of Norit was ashed, another sample from the same bulk was ashed after it had been used to treat nutrient broth, and the metal contents of the two ashes were compared by qualitative emission spectrography. None of the metals showed a decrease after the Norit had been used, but there were increases in the amounts of lead, tin, copper, zinc, silver and cobalt, so one cause of the improvement of broth by Norit treatment could have been that heavy metal ions were removed.

The effect of incubation temperature on the development of E. coli *and* Staph. aureus *in broth media*

Since the constituents of nutrient broth had been shown to contain substances inimical to the survival of bacteria already damaged by exposure to bactericides, it seemed desirable to study the effect of incubation temperature on survival.

Experiments with solid media. Suitable dilutions of normal and phenol treated bacteria were inoculated at random on to standard nutrient agar, and replicates were incubated at 25°, 30°, 37°, 40° and 44° for 72 hr. The mean results are set out in Table 13. With untreated *E. coli* there was no notable effect of temperature on the colony count, though there was a slight reduction at 44°. Untreated *Staph. aureus* grew equally well at 37° and 40° and somewhat less well at 25° and 30°; but at 44° the colony count was sharply reduced. It grew at 44° in only 1 of the four trials and then the count was very small.

Table 13. *The effect of incubation temperature on the counts on standard nutrient agar of normal and phenol treated bacteria*

Incubation temp. (°C)	Mean relative colony count* of			
	E. coli		*Staph. aureus*	
	Normal	Phenol	Normal	Phenol
25	99	63	94	132
30	105	103	91	141
37	100	100	100	100
40	97	48	100	46
44	87	4	0	0

* Relative to 37° values as 100. Normal bacteria, 4 trials with each species. Phenol treated bacteria, 7 trials with *E. coli* and 5 with *Staph. aureus*.

The phenol treated bacteria gave notably lower counts at temperatures above 37° and often no colonies grew at 44°. But while 30° was no better than 37° for *E. coli*, and 25° was definitely less good, both 30° and 25° were better than 37° for *Staph. aureus*.

Experiments with liquid media. Full growth curves could not be established because of the great disparity in the turbidity times at the different temperatures, so a simple form of the dilution method for counting bacteria was adopted. Single drops of treated suspensions were diluted and inoculated into 25 tubes each containing 5 ml of nutrient broth. Five tubes taken at random were incubated for 7 days at each of the five temperatures previously used, and the presence or absence of growth was noted. A colony count was also done at 37° on the suspension from which the tubes were inoculated.

Table 14. *The effect of incubation temperature on the apparent viability of bactericide treated organisms in nutrient broth*

Organism	Treatment of cells	Inoculum level (cells/tube)*	No. of tubes positive† at				
			25°	30°	37°	40°	44°
E. coli	Phenol	1	0	1	1	0	0
		22	5	5	5	0	0
		33	5	5	5	3	0
		60	5	5	5	5	2
		150	5	5	5	5	3
	o-Cresol	11	3	5	5	3	2
		28	5	5	5	5	0
	PCMC	16	4	4	3	0	0
		25	5	5	5	3	0
Staph. aureus	Phenol	22	5	5	5	4	1
		41	5	5	5	5	0
		50	5	5	5	5	0
		61	5	5	5	5	0
		123	5	5	5	5	3
	o-Cresol	30	5	5	5	4	0
		43	5	5	5	5	0
	PCMC	62	5	5	5	5	0
		63	5	5	5	5	0

* Means of 5 replicates on nutrient agar, to nearest unit. † Out of 5 tubes, after 7 days.

The results (Table 14) support those obtained in the previous experiments involving colony counts of survivors. There were fewer positive tubes at 40° and 44° than at 30° and 37°, especially when the inocula gave low counts on nutrient agar. The only indication that 25° was not fully satisfactory was when very small numbers of *E. coli* were inoculated, and even here this may have been due to random sampling.

A further experiment was done, to confirm that the adverse effect of incubation at temperatures above 37° was due to substances in the broth and not to the enhanced action of residual bactericide at the higher temperature. Single drops of a diluted suspension of bactericide treated cells were inoculated at random into 10 tubes each of three media; normal nutrient broth, broth treated with Norit (0·1% w/v) and broth containing added bactericide, the concentration of which was at least 8,000 times that established as the result of carry over with the inoculum. Five tubes

Table 15. *The effects of temperature of incubation on the growth of bactericide treated cells in broths containing low concentrations of phenols‡ and in broth treated with Norit*

Organism and treatment of cells		Inoculum level* (cells/tube)	Numbers of positive tubes† with					
			Normal broth, at		Broth with phenol‡, at		Norit treated broth, at	
			37°	42°	37°	42°	37°	42°
E. coli	Phenol	200	5	5	5	5	5	5
		200	5	5	5	5	5	5
		35	5	2	5	1	5	5
		15	5	1	5	1	5	2
		12	5	1	5	1	5	2
	o-Cresol	23	5	0	5	0	5	1
		30	5	1	5	2	5	5
	PCMC	25	5	0	5	0	5	3
		1	4	0	4	0	5	1
Staph. aureus	Phenol	48	5	3	5	3	5	4
		20	5	2	5	2	5	4
		17	4	2	3	3	3	4
		51	5	2	5	2	5	4
		10	5	1	5	1	5	3
	o-Cresol	8	1	1	1	1	1	1
		12	2	1	2	1	4	2
	PCMC	33	4	4	4	5	5	5
		3	1	0	1	0	2	1

* Phenol treated cells, mean of 5 replicates on nutrient agar at 37°, to nearest unit.

† Out of 5 tubes, after 7 days.

‡ Sufficient bactericide was added to make the concentration at least 8,000 times that present due to carry over with the inoculum.

of each medium were incubated at 37° and the other five at 42°. The latter temperature was preferred to 40° or 44° as it was hoped there would be both positive and negative tubes in all trials. The tubes were examined after 7 days and the results are shown in Table 15. It is clear that whereas the numbers of positive tubes at 42° in the normal broth sets were almost always the same as in the sets with additional bactericide, the numbers of positive tubes at that temperature in the Norit treated sets were almost always higher. This indicates that bactericide carried over in the inoculum could have had little influence on survival. Also, the Norit results confirm that substances present in the medium itself were affecting survival adversely. At 37° the results were less valuable, owing to the frequent occurrence of growth in all five replicate tubes, but the same trend is evident in the results for *E. coli* treated with PCMC and for *Staph. aureus* treated with *o*-cresol and PCMC.

DISCUSSION

The ability of Norit in nutrient broth to reduce the rate and extent of the death in that medium of cells which had been exposed to phenols, and of Norit treated broth to give shorter times to turbidity with such organisms, indicate that the media contained substances inimical to the growth of damaged cells. In experiments involving counts of treated cells suspended in broth containing Norit, the small amount of this adsorbent transferred to the agar counting medium with the inoculum may have removed toxic substances in the neighbourhood of the cells and so encouraged their growth. This point requires investigation. However, the nephelometric experiments with nutrient broth showed that the beneficial effect obtained could not have been due solely to the action of Norit on the nutrient agar. Further, the suggestion of Flett *et al.* (1945) that Norit in media will adsorb phenol and so increase the probability that a phenol treated cell will grow, cannot be supported, since Jacobs & Harris (1954) found that Norit will remove from broth only small amounts of phenol and, as shown above, it will adsorb only few cells under the same circumstances. Moreover the improved recovery of phenol treated cells was also demonstrated in broth which had been treated with Norit and the Norit then removed.

Since Norit, which adsorbed both phenol and cells from water, gave high initial counts when treated cells were added to suspensions of Norit in tap water, this may well be a useful and sensitive technique for testing for the presence of viable cells in suspensions which have been exposed to antiseptics.

It is difficult to understand, however, why the counts of bactericide treated cells suspended in plain nutrient broth should have fallen continuously during standing. Since the nutrient broth used to prepare the agar counting medium was from the same batch as that in which the treated cells were suspended, it might have been expected that the cells would have continued to die after transfer to the agar surface at the same rate and to the same extent as they would have done in the broth, so that a low, constant count representing the final viability level in the broth would have been obtained. Two explanations are possible, that the nutrient agar was less toxic than the broth from which it was prepared, and that the location of cells on the surface of the agar, rather than within the medium, was important. But as to the first of these it is known (Jacobs & Harris, to be published) that agar possesses toxicity independently of the broth and, as some workers have found that toxicity can develop when nutrient broth and agar are autoclaved together (Proom *et al.* 1950; Taylor, 1957), it is reasonable to suppose that nutrient agar would be more, rather than less, toxic than the broth from which it is prepared. As to the second explanation, Taylor (1957) found that the toxicity of agar media to *Bacillus* spp. was exhibited only towards surface inocula and not towards cells embedded in the medium. This possibility has not been resolved in the present work, but experiments have shown that although the species studied are facultative anaerobes, cultivation under anaerobic conditions affected adversely the survival of treated cells.

The weight of evidence thus favours the suggestion that Norit will remove from media substances harmful to cells damaged by phenols; but it is less certain that the pyridine treatment of the medium constituents had a similar effect. The process may have resulted in the removal of nutrients and so have impaired the medium, and as there is some evidence that cells exposed to ionizing radiations survive better under conditions which are suboptimal for the growth of untreated cells (Stapleton, Billen & Hollaender, 1953; Alper & Gillies, 1958, 1960), the possibility cannot be ignored that a similar mechanism was operating in the work reported here. However, the observation that media prepared from pyridine extracted peptone yielded a larger bulk of growth suggests that the removal of toxic substances is the more likely explanation. Also, there was no indication that suboptimal temperatures were favourable to phenol treated cells, although the range did not include the low temperatures which Stapleton *et al.* (1953) found most favourable for their X-irradiated cultures. As to the poor viability observed at temperatures above the optimum, these experiments did not show conclusively that phenol bound to cells was not concerned in this: but it is questionable whether this matter can be resolved.

Isaacs (1930) suggested that organisms exposed to heat or chemicals might be so damaged that a medium on which they grew originally could be unsatisfactory for their recovery. This has become abundantly clear in the present work with phenols: the control medium, which gave good growth of normal cells, was not necessarily suitable for the cultivation of damaged organisms. That such cells are sensitive to subtle variations in the composition of media has been observed by several workers (e.g. Brewer, 1944; Goetchius, 1950) and sensitivity to medium toxicity has been inferred also after cells have been damaged by ultraviolet irradiation (Thompson, Mefferd & Wyss, 1951), by antibiotics (Foster & Pitillo, 1953) and by heat (Olsen & Scott, 1950; Murrell, Olsen & Scott, 1950). But in the present work this situation did not necessarily apply only to damaged cells, since treatments which increased the suitability of a medium for the revival of these also on some occasions increased the bulk of growth obtained in cultures inoculated with undamaged cells. This was especially so with *Staph. aureus*. Further, a parallel situation has repeatedly been shown to exist with undamaged cells of a variety of 'exacting' species, and frequently media have been improved by the use of techniques of adsorption or solvent extraction similar to those which were successful in the present work (Wright, 1933; Glass & Kennett, 1939; Welsch, 1941; Nassau, 1942; Pollock, 1947; Gorelick, Mead & Kelly, 1951; Ensminger, Culbertson & Powell, 1953; Mishulow, Sharpe & Cohen, 1953; Medill & O'Kane, 1954; Jebb & Tomlinson, 1955).

There is little reliable indication of the nature of the toxic material in media. O'Meara & MacSween (1936) suggested that the failure of small inocula of staphylococci to grow was due to the presence of copper in the media and showed (O'Meara & MacSween, 1937) that media free from this metal did not inhibit this organism. They demonstrated also that the effect of copper was neutralized by serum. The wide variations in the copper content of different peptones were noted by Balch (1942) who found also that the inhibitory effects were associated with heating of the medium. A similar phenomenon is referred to also in Report (1956). Elemental sulphur derived from the oxidation of cysteine was found by Schuhardt *et al.* (1952) to be responsible for the inhibition of *Brucella abortus* by some peptones, while fatty acids have been implicated as inhibitors of *Haemophilus pertussis* (Dawson *et al.* 1950, 1951), of heat treated spores of *Clostridium* spp. (Olsen & Scott, 1950) and *Bacillus* spp. (Murrell, Olsen & Scott, 1950). But although charcoal treatment of media can alleviate their toxicity, and charcoal is known to adsorb fatty acids (Linner & Gortner, 1935; Dubinin, 1955), these acids do not appear to be implicated here since starch, which is also known to adsorb fatty acids (Foster & Wynne, 1948; Olsen & Scott, 1950; Murrell, Olsen & Scott, 1950), was not effective in the present work. Nor does the toxicity appear to be of the type observed by Proom, Woiwod, Barnes & Orbell (1950) and by Taylor (1957), where the property developed only when broth and agar were autoclaved together; here the broth itself has been shown to possess toxicity.

The fact that charcoal can adsorb cations (Murkherjee, 1922 and the present work) and the observation that the addition to broth of cations, notably magnesium, promoted the survival of damaged organisms, indicate that toxic cations may be implicated. The literature on the effects on bacterial growth of the addition of cations to media is voluminous. The earlier work has been reviewed by Falk (1923), Buchanan & Fulmer (1930) and Porter (1946), but the results must be regarded with reserve since the presence in media of a wide variety of cations in varying concentrations (Report, 1956) was not then realized. Exposure to phenolic substances is known to result in the leakage of materials from cells (Gale & Taylor, 1947; Bean & Walters, 1955; Haydon, 1956; Beckett, Patki & Robinson, 1958) and this is presumably due to disorganization and increased permeability of the cytoplasmic membrane (osmotic barrier). But this would permit also the easier access of toxic substances to sensitive sites within the cell, and the procedures effective in promoting the viability of damaged cells (Norit treatment of media, the use of pyridine extracted ingredients in the medium and the addition of cations) might all be effective in preventing the access of toxic substances. In the first two cases the effects would be due to their removal from the media, but in the last there could be several effects. There could be competition between the toxic substances and the cations for the sensitive sites (McCalla, 1940; Meggison & Muellar, 1956) and, since cations may reduce the permeability of cell membranes (Shaughnessy & Winslow, 1927), the inward diffusion of toxic substances could be reduced also. In this connection it is noteworthy that Maurice (1952) found that cations reduced the rate of change in optical density of suspensions of *E. coli* in the presence of basic fuchsin and phenol and both Maurice (unpublished data) and Haydon (1956) observed that salts, especially those of metals of higher valency, retarded disinfection by phenol. It is also possible that substances harmful to damaged cells could be removed from media by adsorption on precipitates formed from the added cation or by co-precipitation with them.

The observation of Rubbo, Albert & Gibson (1950) that oxine was harmful to cells only in the presence of cations with which it could complex suggests that complexes between cations and naturally occurring compounds, which Albert (1950, 1952) has shown to occur, could also be toxic. The fact that magnesium, which is known to play an important role in metabolism (e.g. Gale, 1949; Abelson & Aldous, 1950*a*; Webb, 1951), was particularly effective in giving high counts of treated organisms must however be considered in the light of the finding of Mellor & Maley (1948), who observed in an examination involving eight other cations that magnesium gave the least stable complexes (lowest stability constant) with several agents. It could be expected therefore to be displaced from functional complexes in the cell rather than to act as a displacing agent, but it may be suggested that its addition in adequate amounts could cause the displacement of sufficient toxic metals from important sites to enable vital functions to proceed. Support for the present indication of the value of magnesium is provided by the findings of Abelson & Aldous (1950*b*) that this metal antagonized the toxicity for micro-organisms of nickel, cobalt, cadmium, zinc and manganese, and by the suggestion of Taylor (1952) that cations (notably calcium but also strontium and magnesium) can antagonize growth inhibitors in peptones.

In conclusion it may be remarked that in all probability many factors are involved in medium toxicity. The extent to which each factor influences a given system could depend on the species of organism, the type of damage, if any, and on the exact composition of the medium, not only nutritionally but also in the nature and concentration of both toxic substances and their antagonists.

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The present address of Dr. N. D. Harris is: School of Pharmacy, Chelsea College of Science and Technology, London, S.W.3.

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(Symposium on Methods for the Evaluation of the Antibacterial Activity of Surface Active Compounds: Paper I)

METHODS FOR THE EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF SURFACE ACTIVE COMPOUNDS: TECHNICAL ASPECTS OF THE PROBLEM

By J. G. DAVIS

Dr. J. G. Davis & Partners, 9, Gerrard Street, London, W.1

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1. Introduction and Definitions

Surface active agents may be classified as (1) anionic, (2) cationic, (3) non-ionic, and (4) amphoteric or ampholytic. In our experience the first and third groups have strong detergent but negligible bactericidal powers, and the second strong bactericidal but negligible detergent powers at ordinary use dilutions (100–1,000 p/m). The fourth group has fair detergent powers. The germicidal powers have not been extensively investigated, but very strong activities have been claimed.

In the following review certain terms have been used in a particular sense, which it has been thought desirable to define. Thus a germicide is any substance or combination of substances capable of killing bacteria; the term disinfectant connotes a powerful germicide with no specific qualifications; an antiseptic is a germicide which has no irritant effect on animal tissue under specified conditions of use, and a detergent-sanitizer is a preparation having both detergent and germicidal properties under specified conditions of use. The detergent power may be due to alkalis or organic substances or both (Soc. Dairy Tech. 1959, p. 51; Neave & Hoy, 1947; Garvie & Clarke, 1955; Clegg, 1955; Schwartz *et al.* 1958).

2. Types of Surface Active Germicides

(a) *Cationic germicides*

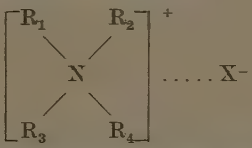
This term today usually implies a quaternary ammonium compound (QAC). For all practical purposes these compounds are nontoxic, odourless, noncorrosive, harmless to handle, sufficiently soluble, effective over a wide pH range, highly penetrative, persistent in their action and particularly effective against Gram-positive organisms. Moreover, they are nonirritant; for example, the threshold concentration causing inflammation in a rabbit's eye is about 1:330 (Davis & Resuggan, 1946).

Their disadvantages are few. They are rather expensive, less effective against Gram-negative organisms, and some types readily foam; but nonfoaming types are available (Resuggan & Davis, 1947).

They are not easily rinsed away: but this persistence is advantageous and the traces remaining on food equipment are not of any public health significance.

(i) *Historical.* Hexamethylenetetramine was introduced as an internal antiseptic by Nicollaier as long ago as 1894, and Einhorn & Göttler recognized the germicidal properties of the chloroacetylaminomethanol derivative of hexamethylenetetramine in 1908. In 1913 Reychler described the synthesis and properties of cetyltriethylammonium iodide, and Jacobs (1916) and Jacobs *et al.* (1916*a*,*b*) studied the relation between the chemical constitution of the quaternary hexamethylenetetramine salts and their bactericidal properties. Little further interest was taken in these compounds, apart from Hartmann & Kägi's paper in 1928, until 1935, when Domagk emphasized the strong germicidal power of alkyldimethylbenzylammonium halides. Many papers on medical applications followed in rapid succession, and in the 1940s many papers appeared in which applications in the food industry, catering, public health, dairying, brewing and other fields were dealt with.

All QACs may be regarded as members of the group



The radicles R_1, etc., may be short, medium or long paraffin chains, or simple or substituted benzyl or heterocyclic radicles such as pyridinium. Detailed information on the many known QACs is given by Glassman (1948), and Lawrence (1950) and Resuggan (1951) have discussed their application in the food industries. The most recent collected information on these compounds is given by Reddish (1957) and Schwartz *et al.* (1958).

(ii) *Physical properties.* The QACs ionize to give a large positively charged ion and a small negatively charged hydrophilic ion. Compounds of this type have a tendency to form micelles (McBain, 1913; Ralston *et al.* 1942; Valko, 1946; Ralston, 1946) and micelle formation may account for anomalous behaviour (Adam & Shute, 1938). With dilute solutions, where there is little micelle formation, the final surface tension may not be reached for a week.

It has been suggested that bactericidal power is related to surface activity (Suter, 1941; Cowles, 1938), but many surface active compounds have negligible germicidal activity. The peculiar properties of QACs and their much greater 'killing' effect against Gram-positive organisms support the theory of a specific reaction with constituents of the cell wall.

(b) *Anionic germicides*

Soaps. Although soap alone is not generally considered a strong germicide, it is effective under certain conditions against some organisms (Walker, 1924, 1925, 1926), and a 0·25% soap solution may be more effective against streptococci than 0·7% Lysol or 0·1% mercuric chloride (Colebrook & Maxted, 1933). However, the greater value of soap, as of all substances having detergent properties, lies in its power to remove adhering organic matter which may serve as a matrix for bacteria amounting to millions/g, and from the numerical standpoint a detergent may be more valuable in removing bacteria mechanically than an accompanying disinfectant (or disinfectant power) in killing them. However, simple detergent efficiency is difficult to measure and it is usually not possible or necessary to assess these two effects separately.

Soaps are pre-eminently of interest from the point of view of cleansing the human skin (this term covers the removal of adhering matter, the killing of pathogens and the reduction of the bacterial numbers to a very low level), and the assessment of the usefulness of a soap for this purpose is a complicated problem (cf. Bettley, 1960).

Phenol-soap mixtures. Although soap by itself may have only weak germicidal, as distinct from detergent, properties it has the power of solubilizing all types of phenolic compounds, including the strongly germicidal cresols and chlorinated cresols (Engler & Dieckhoff, 1892; Bean & Berry, 1950; Berry, 1952; Finch, 1958). From the bacteriological point of view these mixtures may be regarded as phenols with detergent properties, and most of the tests which are suitable for measuring the activity of phenol should be suitable for them.

Other anionic compounds. The only other types of any consequence are the sulphated alkyl and sulphonated aryl compounds. Some of these may be useful against Gram-positive bacteria, but almost all are ineffective against Gram-negative types. In addition the effective pH range is limited. The members of this general group of anionic detergents are invaluable as cleaners but of little value as germicides.

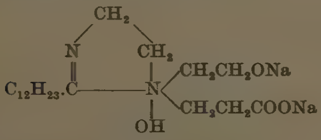
(c) *Non-ionic germicides*

Although non-ionic detergents are without any significant germicidal power they can be compounded with iodine to give the iodophors, which are both surface active and powerful germicides. In the latter respect they behave similarly to iodine but without the staining and other disadvantages of that substance. They have received some attention in America (Johns, 1954) and are now being tried in this country (Cousins *et al.* 1959), particularly in the dairy industry. Both from the use and testing points of view they may be regarded as iodine. However, micelle formation may affect activity as with the QACs (cf. Allawala & Riegelman, 1953).

(d) *Amphoteric (ampholytic) germicides*

The members of the fourth group, although known chemically for many years, are relative newcomers in the disinfectant field. Strong claims have been made for their germicidal powers, but little is known of them in this country and they are not yet in general use.

Table 1. *Typical amphoteric (ampholytic) surface active germicidal agents*

Chemical name or formula	Trade name	Manufacturer
Dodecyl-β-alanine	Deriphet	General Mills
Dodecyl-β-aminobutyric acid	Armeen Z	Armour
N-Lauryl-aminosulphonic acid	Siposan	Sipon
	Miranol	Miranol Chemical
Dodecyl-di(aminoethyl)-glycine	Tego	Th. Goldschmidt

One of the first workers to stress their possibilities was McCutcheon (1955), who described the properties of dodecyl-β-alanine. The ampholytics are not necessarily confined to carbon and nitrogen compounds, but only these appear to have been used so far. They are based on two main types, viz. long chain N-substituted amino acids and long chain betaines.

Solutions of the alkylaminopropionic acids may have very low surface tensions and are therefore good wetting agents. They form micelles at all pH values and are fairly good emulsifiers. Like the QACs they are strongly adsorbed at low pH values. They are compatible in all forms with anionic, non-ionic and cationic agents, with the sole exception that the acid salt is incompatible with anionics. Typical examples of these compounds, with their trade names and manufacturers, are given in Table 1.

Although accepted as ampholytic, the betaines are really internal QACs and Moore (1960) suggested that these should be called *intronium* surface active agents. While in some respects they resemble QACs, their general physical properties are very similar to those of the alkyl amino acids.

Compared with the anionics and non-ionics (which have good detergent but poor germicidal properties) and the cationics (with poor detergent but good germicidal properties), the amphoterics have good detergent and, it is claimed, good germicidal properties. The critical micelle concentration is 0·2–0·4 g/l., which may be compared with the level of 1–2 g/l. for the anionics and the cationics (Schmitz & Harris, 1958). Owing to their dipolar nature they may also form aggregates by 'internal desaturation' (McCutcheon, 1955).

3. Types of Test for Antibacterial Activity

Two of the most important methods involve determining the concentration necessary to kill a given number of cells in a given time or measuring the rate of kill by making colony counts at intervals. Another type of test is that in which a zone of inhibition of growth of the test organism is produced, and finally there are methods in which activity is measured in terms of the inhibition of some metabolic activity by the germicide. These four kinds of method are briefly considered below.

(a) *Methods involving 'complete kill'*

It is obvious that these methods are influenced by the concentration of cells in the test solution more than the methods depending on rate of kill, as the higher the initial number the longer the time or the greater the concentration required to give a 'complete kill'. But since the criterion of a 'complete kill' is usually the failure of a small volume (e.g. 1 loopful) of the mixture of cells and disinfectant to initiate growth when inoculated into a suitable medium the effect of random sampling becomes important and in practice these methods (sometimes called 'end-point' methods) probably measure something less than 100% kill. The extent of the difference depends on the volume subcultured and the number of replicate tests as well as on the general conditions in the test.

In all tests where the presence of survivors is judged by the appearance of turbidity in the subcultures, care must be taken to verify that the turbidity is actually due to growth. Lower concentrations of a QAC which forms a precipitate with constituents of a culture medium may produce a turbidity when higher concentrations do not because sedimentation occurs. Also the germicide solution itself may be turbid, and soap solutions may vary in turbidity (Bean & Berry, 1951).

(i) *The phenol coefficient.* In spite of the many criticisms that have been made the phenol coefficient method (Rideal & Walker, 1903; Ruehle & Brewer, 1931; Specification, 1934; McCulloch, 1945; Reddish, 1957; Finch, 1958; Sykes, 1958) is still used all over the world for measuring the disinfectant power of germicides. It has the substantial advantage that a simple numerical value related to the behaviour of a pure chemical substance is obtained. In this method the errors associated with the type and physiological condition of the test organism are supposedly standardized, but there are important limitations to its use with QACs. These are discussed in detail in section 5.

(ii) *Critical killing dilution.* In this, the highest dilution (lowest concentration) capable of killing a suspension of bacteria under standardized conditions is determined. As with the phenol coefficient method it has the advantage that the result can be expressed as a simple and easily understood number. For cetylpyridinium chloride Quisno & Foter (1946) obtained values varying from 1:1500 with a *Mycobacterium* sp. to 1:127,500 with *Strep. pyogenes* in the absence of serum, and from 1:1000 to 1:20 000 respectively in the presence of 10% of serum. In similar tests a number of QACs gave limiting dilutions varying from 1:1600 to 1:200,000 (bactericidal) and from 1:4800 to 1:800,000 (bacteriostatic) (Lawrence, 1950, p.72). Bactericidal were distinguished from bacteriostatic effects by making transfers from the test cultures to fresh tubes or plates.

As one method of removing residual germicide Nagel (1940) washed the cells after treatment. With this technique not only staphylococci and streptococci but also coli-aerogenes bacteria were destroyed in 1 min by 0·25% QAC, and *B. subtilis* spores were killed in 15 min by 1–2%; but *M. tuberculosis* was not killed in 24 hr by 2%.

(b) *Methods involving rate of kill*

(i) *Suspensions.* The simplest basic test for disinfectants is to allow the substance to act on a suspension of bacteria under standardized conditions and to measure the rate of killing by colony counts or some other suitable method at specified time intervals. This was the method used in the classical disinfection studies (Chick, 1908, 1910, 1921; Phelps, 1911; Withell, 1942; Jordan & Jacobs, 1944, 1945). While this method will remain the choice of research workers, it is of limited value to the user who needs to know whether a germicide used as prescribed will sterilize a given piece of equipment, i.e. give a 100% kill, under the worst conditions which occur in practice. However, it is in fact doubtful whether 100% kill is necessary for a germicide to be effective in practice. Tests are usually made with at least 10^6 cells/ml, a far higher number than is usually encountered in practice, e.g. on detergent cleaned utensils or on washed skin. Moreover, even if a few surviving bacteria can grow under the optimum conditions provided in disinfectant tests, e.g. in broths at 37°, it does not follow that all of these would proliferate on the utensil or on the skin after removal of the germicide. This aspect is of special significance for QACs and other germicides which display a 'persistence effect'.

Finally, it is well recognized that a minimum number of viable cells, which may be relatively large, is needed to start an infection or cause a defect; the probability is that no one has ever suffered harm from a few isolated bacteria surviving from a germicidal treatment. Special cases such as that of *M. tuberculosis* are not included in this generalization as they are outside the present field of enquiry, but even here a minimum number of cells, e.g. 100, may be necessary to establish an infection.

If this approach to the problem is accepted it follows that a 99·99% kill in a short time under standardized conditions can be accepted as a satisfactory criterion. This has the advantage that the required bacterial counts are conveniently made. Also, the result is not invalidated by the occurrence of the occasional and apparently inevitable 'freak survival'. If 100% kill is adopted as a criterion such survivals complicate the testing.

(ii) *Surface films.* In practice it is usually the destruction of bacteria in films of organic matter on surfaces, e.g. of tissues, metal, glass and textiles, which is required and it was early realized that suspension tests, although relatively easy to perform and standardize, were of limited value in predicting the efficiency of a germicide used for sterilizing skin, milking equipment and blankets (Clegg, 1955). Of the various factors involved, the physiological condition of the bacterial cells and the possible protection afforded are probably the most important. For example, it is much easier to kill a suspension of a 24 hr broth culture than the bacteria in a dried milk or serum film 3 days old. In the latter case the cells are static and protected by a firm film of milk or serum solids. The detergent or disintegrating power of a detergent-sanitizer is then of equal importance to its germicidal power. In practice the effect produced depends on both detergency and germicidal action. Detergency is such a complicated process that no simple test is available for assessing it, but though a film test really measures two effects the method is sound from a practical point of view. Whether the bacteria attached to a surface by dried organic matter are killed or merely removed and washed away is usually immaterial in the food industry. In medicine, or when working with pathogens, the killing process can still proceed after the germicidal solution has ceased to act on the surface concerned. In surface film tests factors inoperative in suspension tests may come into play. Thus Garvie & Clarke (1955) found that humidity affected the proportion of survivors in tests with *E. coli* but not with *Staph. aureus*. More organisms survived in films than in solutions.

In the 'use-dilution' method of Mallmann & Hanes (1945) the test organisms are dried on glass rods for 30 min, exposed to a solution of the disinfectant for 1, 5, 10 and 30 min, the rods rinsed with water or an inactivating solution and then placed in culture media. This method is stated to be satisfactory for QACs. Jensen & Jensen (1933) used cover slips, and Klarmann & Wright (1946) glass slides.

A similar technique is the glass slide method of Johns (1947) in which a suspension of the test organism in 10% skim milk is applied to slides, dried, immersed in the solution, rinsed and then placed in an agar medium. Stuart (1947) used glass rings.

Basically similar tests using metal equipment more closely related to practical conditions have been developed by Meewes (1941), Neave & Hoy (1947), Hoy & Clegg (1953), Clegg (1955), Garvie & Clarke (1955), Hirsch & Muras (1955), Garvie

(1957) and Whitt (1958). Goetchius & Botwright (1950) used rubber strips, which are notoriously difficult to sterilize.

(c) *Agar diffusion methods*

These methods have become popular, not only as screening or sorting tests for disinfectants but also as standard methods for the assay of antibiotics. The disinfectant or antibiotic is allowed to diffuse from a cup cut in an inoculated agar plate or from a cylinder placed on its surface and produce a zone of growth inhibition. Alternatively impregnated filter paper discs may be used (Vincent & Vincent, 1944). There is usually a linear relation between the logarithm of the concentration of disinfectant and the diameter of the zone of inhibition. Standardization of all details is essential if reasonably reproducible results are to be obtained. Thus the time and temperature at which the agar plates are hardened can have appreciable effects on the results (Davis, unpublished data).

(d) *Methods involving inhibition of metabolic activities*

Numerous methods, based on the inhibition of some easily measurable metabolic activity such as respiration, reducing power or fermentation, have been published. While of considerable fundamental interest such methods are even farther removed from practical applications than those depending on a mathematical treatment of survivor counts.

Some of these methods depend directly on the nature and extent of the damage done to the cell by the disinfectant. Thus in the method of Maurice (1952) the opacity changes in a mixture of cells, dye and germicide depend on the alteration of the permeability of the cells by the germicide. Manometric metabolic methods may be suitable for QACs but not for other types of germicide (Hugo, 1952).

4. FACTORS AFFECTING TESTS FOR ANTIBACTERIAL ACTIVITY

It is now generally agreed that the only completely satisfactory way to test germicidal preparations is to use them under the actual conditions in which they will be used in practice. Even then the method of assessment and the end point must be chosen arbitrarily; or alternatively their efficiency may be evaluated in terms of prevention of infection or of a satisfactory keeping quality for a food. However, laboratory tests can be very useful for 'sorting' purposes provided they are properly designed. The nearer the laboratory test conditions are to the conditions of use the more reliable will the results be. Practical conditions can never be standardized because they vary from day to day; hence laboratory tests should be regarded as giving information complementary to that obtained by practical use tests.

The following factors may affect the result of any test on a disinfectant.

(a) *The test organism*

(i) *Choice.* Apart from the few well-known exceptions, infections and food spoilage are caused by nonsporulating, non-acid-fast organisms which are relatively susceptible to disinfectants and antiseptics.

Few organic compounds are effective against all types of micro-organism, but surface active germicides especially have a restricted 'spectrum' of activity, often being highly toxic to some bacteria and relatively innocuous to others. It follows that the choice of test organism is of great importance. The one chosen should be as appropriate as possible to the problem.

From the testing aspect the public health interest appears to be greater than the food spoilage interest. Most of our work has been done with the former aspect in mind, and the following representative organisms have been used. Gram-positive; *Staphylococcus aureus*, *Streptococcus pyogenes* and *Strep. faecalis*: Gram-negative; *Salmonella typhi*, *Escherichia coli* I and *Pseudomonas aeruginosa*.

In our experience all QACs are so effective against Gram-positive organisms that there is little point in trying to assess degrees of effectiveness. The Gram-negative species are almost invariably more resistant; *Salm. typhi* and *E. coli* behave similarly, but *Ps. aeruginosa* is appreciably more resistant than these and in practice any treatment by a QAC preparation which is satisfactory against the latter can be relied upon to be effective against the other five. *Strep. pyogenes* is usually the least and *Strep. faecalis* the most resistant of the Gram-positive cocci, but from the point of view of potential danger *Staph. aureus* is the most appropriate test organism. However, *Strep. faecalis* is likely to give more reproducible results as it does not form clusters as does *Staph. aureus*. If *Ps. aeruginosa* is not important *E. coli* can safely be accepted as the test organism as it is invariably more resistant than Gram-positive organisms (excepting spores) and is usually more resistant than *Salm. typhi*. Typical results are given in Table 2. With soap-phenol mixtures Berry (1952) concluded that *E. coli* was the most suitable for weak preparations and *Ps. aeruginosa* for strong ones. The latter was actually more resistant to phenol than heat resistant spores, an illustration of the fact that germicide resistance and heat resistance are fundamentally different properties.

Table 2. *The survival of different organisms in wash liquor (dish-washing) containing a non-ionic detergent and a QAC*

(Davis, unpublished data)

Organism	Colony count/ml after (min)				
	0	1	2	3	4
Ps. aeruginosa	52,000	4,900	2,920	2,360	2,290
E. coli	3,100,000	42,000	40	<10	<10
Salm. typhi	1,180,000	600	10	<10	<10
Staph. aureus	870,000	<10	<10	<10	<10

Broadly speaking, the QACs are much more effective against Gram-positive bacteria than against Gram-negative bacteria or yeasts, and moulds are more resistant than yeasts. They are virtually useless against spores (Davies, 1949), a concentration of 10,000 p m being necessary to kill those of *Bacillus subtilis* in 4 hr (Steingold *et al.* 1954).

In chemical sterilization procedures in the public health field *Mycobacterium tuberculosis* still remains the greatest problem. Claims for powerful germicidal activity against this organism appear regularly for all types of compounds, but in our experience these claims are rarely substantiated. Together with *Ps. aeruginosa*, *M. tuberculosis* may be regarded as the test organism *par excellence* for any new germicide for which extraordinary claims are made. Claims for effectiveness against *M. tuberculosis* have recently been made for some of the ampholytic compounds; for a new member of this group one manufacturer has claimed a Rideal-Walker coefficient of 37 for a 50% preparation.

(ii) *Origin and condition.* In standard methods the use of a certain culture from a recognized collection is usually specified; but such cultures may change their characteristics with age, and laboratory maintained cultures may be less resistant than freshly isolated strains from infections. We recommend, therefore, that a freshly isolated strain, preferably from an infection or faulty product, should be used as well as a type culture in disinfectant testing.

Physiological condition is usually standardized by using a 24 hr broth or agar slope culture after three serial 24 hr subcultures at 37° or other suitable temperature. The culture medium is also standardized, but materials such as peptone, even when specified by name, can never be guaranteed to be constant in behaviour. If a suspension is prepared from an agar slope culture by washing the cells off with Ringer's solution they may be weakened (cf. section 4, d vi), but this technique gives a suspension relatively free from medium constituents. Direct addition of a broth culture avoids this washing of the cells but involves the addition of a small amount of medium to the test solution.

Errors can also arise from clumping of the cells, which occurs readily with such species as *Staph. aureus*. Adsorption of a surface active germicide on such clumps may help to prevent their disintegration and so protect the inner cells from the germicide. Bean & Berry (1951) observed that soap solutions tended to clump bacteria, especially *Ps. aeruginosa*. Colony counts also will be affected by the degree of clumping.

(iii) *The initial number of cells.* Apart from any specific interaction between germicides and constituents of bacterial cells, the cells themselves behave as organic matter and so will tend to weaken disinfectant action. It is therefore necessary to standardize the concentration of cells either in the medium or suspension or on the surface of the test object.

A convenient initial number of viable cells (units forming colonies) for standardization purposes is 10^6/ml of medium, or of rinse if surfaces are to be disinfected. This number is not large enough to introduce organic matter effects or to favour clumping, but is sufficient to provide a reasonable count for a 99·99% kill. It is advisable therefore to work with an initial count in the range $0{\cdot}5\text{–}2{\cdot}0 \times 10^6$/ml. Some workers have found that reproducibility suffers if initial counts appreciably higher than 10^6/ml are used. However, if a 99·999% kill is adopted as the criterion of effectiveness, a higher initial count is necessary. Thus Ortenzio (1958) suggested an initial count of $75\text{–}125 \times 10^6$/ml.

(iv) *The nature of the recovery medium.* Of the six organisms mentioned above only *Strep. pyogenes* is particularly fastidious in its growth requirements. Selective media are available for all six cultures but they tend to be inhibitory even to the organism in question and there is no special advantage in using them in disinfectant testing. Media permitting luxuriant growth of the test organism may, however, not only give damaged cells the best opportunity to recover (see e.g. Jacobs & Harris, 1960), but by allowing contaminants to grow they automatically provide a continual check on technique.

For routine testing we use a medium containing: peptone, 5 g; Yeastrel, 5 g; glucose, 5 g; NaCl, 5 g; milk (whole), 10 ml and tap water (known satisfactory), 1,000 ml. This solution is adjusted to pH 6·8, autoclaved momentarily, filtered, tubed and autoclaved. For solid media agar (15 g) is included. Suitable modifications to this basic medium can be made for particular organisms. With *Strep. pyogenes* 10% of serum is added at the time of plating or culturing.

All media constituents must be checked for suitability; this is normally done automatically in frequent testing, any anomalous behaviour of any organism on a new batch of medium being immediately recognized by an experienced worker. When special media constituents are prescribed for specific tests, e.g. the Rideal-Walker coefficient by the Crown Agents' method, these should be used. However, it seems unnecessarily complicated to recommend different peptones for similar tests, and simplification of all official methods in this respect is highly desirable.

(b) *The environment of the test organism*

(i) *The chemical quality of the water.* Hard waters may considerably reduce the bactericidal power of QACs (Table 3).

Table 3. *The effect of the nature of the water on the bactericidal action* of three QACs*

(Data from Report, 1947)

QAC	Lethal concn. (p/m) when used in			Use concn.† (p/m)
	Distilled water	Cincinnati water	Norwood water	
A	20	100	250	234
B	10	70	220	190
C	5	500	3,200	78

* Concentration giving 100% kill of *E. coli* in 1 min. † Recommended by the makers.

It was found that pH, organic matter, anions and monovalent cations had no significant effect, but 40 p/m of calcium or magnesium could reduce the kill against *Salm. typhi* from 99·9% to 50%. However, this hardness effect is not generally apparent with Gram-positive organisms (Johns, 1948; Lawrence, 1950, p. 36). The adverse effect of calcium salts may be removed by adding small quantities of alkaline detergents such as trisodium phosphate. Cousins & Clegg (1956) emphasized that not only do QACs vary in their behaviour in hard waters, but the effects of added detergents also vary. Nothing can be assumed.

(ii) *The effect of pH.* This can be considerable. It varies with the particular QAC but bactericidal effect usually increases with increasing pH (Dunn, 1937; Gershenfeld

& Milanick, 1941; Gershenfeld & Perlstein, 1941). Typical results are given by Lawrence (1950, p. 78). However, there are many anomalies (cf. Hucker *et al.* 1948) and the pH effect may vary according to the organism used (Salton, 1950; Soike *et al.* 1952). In the region of pH 4, QACs become relatively ineffective, but cetylpyridinium chloride appears to be an interesting exception (Quisno & Foter, 1946). It should not be forgotten that the pH of a solution may change on standing (Stuart, 1947).

It seems that the nature of the buffer may be at least as important as the pH itself (Clarke & Garvie, 1955), a point frequently overlooked in both bacteriological and biochemical work. The concentration of other ions is usually altered even if only one buffer is used, and if different buffers are used in pH range experiments at least two other variables are introduced. It is impossible to alter a solution in pH alone.

(iii) *The degree of aeration.* In a laboratory test the degree of aeration is probably fairly constant, but under practical conditions, especially where large volumes are concerned, the oxygen tension may vary and could conceivably affect germicidal power against obligate aerobes and perhaps even against facultative anaerobes.

(iv) *Temperature.* The rate of killing invariably increases with temperature, but different QACs possess widely different temperature coefficients. Thus two QACs may be significantly different in effectiveness at 20° but about equal at 40° (Cousins, 1951).

Many disinfection tests are made at 20°, though a temperature of 37° may be used, especially with antiseptics. Bearing in mind that detergent-sanitizers are invariably used at least warm we have standardized on a temperature of 45° for the reason that this is the highest temperature for the comfortable hand-washing of dishes and equipment in general.

(v) *Organic matter.* It has long been known that disinfectants are greatly weakened in the presence of organic matter (Chick & Martin, 1908). Although QACs may be less sensitive than other disinfectants (Domagk, 1935; Hornung, 1935; Kliewe & Maier, 1936) their germicidal powers are invariably weakened. One well known illustration of this effect is the fact that the spread of Rideal-Walker coefficients is much greater than that of Chick-Martin coefficients. In other words organic matter tends to level out differences between disinfectants. Cousins (1951) observed that inconsistencies in survivor counts were reduced when milk was added to the test solutions.

A good example of how organic matter can nullify the results obtained by orthodox *in vitro* tests is afforded by the performance of mouth washes. The phenol coefficient test is virtually useless (American Dental Association, 1932). Welch & Brewer (1942) found that out of 87 proprietary mouth washes only 9 possessed any appreciable germicidal power in use. Wolf & Brown (1946) found that in as short a time as 10 min after the use of a mouth wash the mouth flora showed little change, and it was back to normal after 1 hr.

It can be expected that the greater the amount of organic matter, and the more firmly it is attached to the equipment or tissue being 'sanitized', the more important is the detergent power of any sanitizing preparation. Thus Wolf & Brown (1946) observed that of all the preparations tested, which included some very powerful *in vitro* germicides, the most effective was 1% soap solution which, by *in vitro* tests, would be rated as very feeble.

It is an easy matter to incorporate a known amount of organic matter in any test. The obvious material to choose is that which is normally present under the conditions of use, e.g. serum, blood, faeces, urine, milk, or even excess of the cells of the organism to be killed. Constancy of composition is essential and in practice availability and convenience are also important factors. For standardization purposes there is much to be said for selecting one such material, and after trying nearly all the usual soiling materials we have come to the conclusion that bulk whole milk is the best. The five reasons for this decision are that it is practically constant in composition, it contains fat, protein, sugar and salts in almost constant proportions, it is universally available at all times, volumes can be rapidly measured, and it is more protective than most other organic materials which have been used for this purpose.

Much of our work has been in relation to dish washing, which is probably a typical operation from the points of view of use, type of soiling material and time of treatment. We have found that dishwashing solutions contain when discarded after use about 0·5% of soiling matter on a dry weight basis, and we have therefore standardized on an addition of 4% of whole milk (0·5% dry matter) in our disinfectant testing. Typical results are given in Tables 4 and 5. For antiseptics albumin is suitable and convenient and the concentration may be varied appropriately; e.g. for mouth wash tests it should correspond to the protein content of saliva.

Table 4. *The effect of organic matter on the bactericidal powers of three non-ionic detergent QAC mixtures against* Salm. typhi

(Davis, unpublished data)

Addition	Kill (%) with detergent-QAC mixture no.		
	1	2	3
None	99·998 99·852 >99·999	99·975 99·999 99·985	99·985 99·961 99·922
Peptone (0·5%)	99·998 99·992 >99·999	99·997 99·840 99·970	90·9 99·977 99·961
Milk (4%)	99·967 99·995 99·999	75·2 72·2 70·0	83·2 88·1 86·2

The effect of organic matter depends not only on its nature and concentration but on the test organism, the QAC in question, temperature, time and other factors. Therefore, while we suggest that bulk whole milk is probably the best for standardization purposes, we emphasize that parallel tests with the relevant organic matter should also be made.

(vi) *Osmotic pressure.* Most disinfectant tests are made with very dilute solutions of germicide and as organic matter, even at a 2–4% level, does not affect osmotic pressure significantly it is unlikely that differences in osmotic pressure encountered in practice will affect the result. However, in special techniques consideration of this factor may be required.

(vii) *Detergent-sanitizers.* Many QAC preparations are now sold as detergent-sanitizers. These generally consist of QACs compounded with non-ionic detergents or mild compatible alkalis, or both. Such preparations are tested at the dilution recommended by the manufacturers, e.g. 1:300. Two of the major factors affecting the tests, pH and concentration, are therefore automatically standardized.

During their use, e.g. for washing dishes, the disinfectant action of the solution steadily decreases for four reasons. First, there is loss of QAC through 'carry-over' and/or dilution (up to 50% or more). Second, the organic matter content increases (from 0 up to 0·5% dry matter or more). Third, there is a fall in pH, e.g. from 11 to 10 and fourth, the temperature drops, e.g. from 120° to 100°F. It follows that any test made on a fresh solution gives only part of the vital information. In assessing the value of any cleansing preparation (i.e. one which it is claimed will both clean and sterilize) tests should always be made on the solution at both the beginning and the end of a run (cf. Resuggan & Davis, 1947; Davis *et al.* 1949).

While the general public usually thinks of the sanitizing process chiefly in terms of the killing of the bacteria by chemicals, it should be remembered that 90–99% of the bacteria are removed from the equipment by the cleaning process. Chemical disinfection is only a 'finishing' process.

When testing the effectiveness of detergent-sanitizers either the wash water itself, as in dish or glass washing, may be plated, or the utensils, e.g. dishes or glasses, may be swabbed. Both methods give results of the same order (Davis & Resuggan, 1946).

Table 5. *The effect of organic matter and alkali on the bactericidal activity of four QACs under standard conditions*

Organism	Additions	Kill (%) with QAC* no.			
		1	2	3	4
Staph. aureus	None	>99·997	>99·995	>99·997	>99·996
	Milk (8%)	>99·995	>99·994	>99·950	>99·995
	Na_2CO_3 (0·2%)	>99·994	>99·993	>99·996	>99·991
	Milk (8%) + Na_2CO_3 (0·2%)	>99·983	>99·967	>99·950	>99·950
E. coli	None	98·300	99·000	99·540	99·890
	Milk (8%)	88·500	87·300	83·200	82·200
	Na_2CO_3 (0·2%)	99·995	99·999	>99·999	99·99
	Milk (8%) + Na_2CO_3 (0·2%)	99·999	99·993	96·400	83·000

* The QAC concentration was always 1:13,710.

As might be expected, incorporation of an alkaline detergent greatly increases the effectiveness of most QACs, not only against Gram-positive bacteria but especially against *E. coli* and other Gram-negative organisms (Table 5).

Considerable attention has been paid to the testing of detergent-sanitzers in the U.S.A. and a standard method, based on collaborative studies, has been reported on by Ortenzio (1958). A reduction of 99·999% in 30 sec at 25° is required (see section 4, a, iii).

While the addition of ordinary alkalis (NaOH and Na_2CO_3) may enhance the bactericidal power of most QACs, sufficient residues of known incompatible detergents (section 4, d, iv) may be present under practical conditions seriously to reduce the germicidal effect of subsequently applied sterilants. Thus soap residues may inactivate QACs, and non-ionic detergent residues may inhibit phenolic germicides. A point often overlooked is that alkali-treated fat-soiled surfaces may retain soaps (resulting from hydrolysis) and so reduce the efficiency of a QAC. This effect does not occur in the absence of fat (Ortenzio *et al.* 1954).

(c) *Inactivation*

All tests of disinfectants involve action for a defined time, and good technique requires that the time shall be measured with sufficient accuracy, e.g. ±1%. With methods involving withdrawal of an aliquot after a few minutes and examination by a plate count or for surviving cells, it is obvious that the germicide is withdrawn with the suspension and continues to act, even though diluted 10 or more times. The simplest way to stop the action of the disinfectant is to add an innocuous substance which neutralizes its action or destroys it. Commonly used materials are named in Table 6. The times of reaction of these are obviously different; only a few are virtually instantaneous in action.

Table 6. *Substances commonly used as inactivators of germicides*

Germicide	Inactivator
Chlorine or hypochlorite	Sodium thiosulphate
Formalin	Ammonia
Phenols	Charcoal; ferric chloride
Hydrogen peroxide	Catalase
Mercurials	Sodium thioglycollate
Mercuric chloride	Sodium sulphide
QACs	Lecithin; stearate; Lubrol W
Sulphonamides	*p*–Aminobenzoic acid
Penicillin	Penicillinase
Streptomycin	Glucose
Hexachlorophene	Tween 80 (Lawrence & Erlandson, 1953)

It is well recognized that QACs may be adsorbed to bacterial cells and prevent them from growing, but not kill them during the test period. Removal of the adsorbed QAC within 10–30 min may result in resuscitation of the cell (Valko & Dubois, 1944). However, this phenomenon is highly variable, and depends on the type of bacterium used, the QAC and the inactivator. There is no clear cut distinction between bacteriostatic and bactericidal action, or between ineffective disinfection and 'reversible dying' (cf. Marshall & Krenoff, 1937; Price, 1950; Cook, 1954). But it has become standard practice to add an efficient inactivating agent to the disinfection mixture at the end of the test period. Lecithin (Baker *et al.* 1941*b*), stearate, Lubrol W, suramin sodium (Lawrence, 1947, 1948) and aryl sulphonic acid condensate (Goetchius, 1948) have been used, and even a natural substance such as milk in high concentration. Meanwell (pers. comm.) found that 10% of milk was satisfactory for inactivating QACs in tests with *E. coli*. Such compounds may revive Gram-negative but not Gram-positive bacteria (Klein & Kardon, 1947) and this procedure obviously effects an artificial distinction between cells which are already killed or irretrievably dying, and those which would die if the adsorbed QAC were not inactivated.

When QACs are used in practice we do not immediately treat the bacterial cells by this inactivation procedure. It follows that those cells which are 'reversibly dying' will die some time after the end of the treatment period, e.g. 10 min, and can therefore hardly be considered dangerous to health. It may be debated which picture—the result with, or the result without inactivation—is more closely related to practical applications of quaternary germicides (cf. Rahn, 1947).

(d) *Other factors*

(i) *Errors in measuring.* Volumetric measurement of any solution of a surface active substance is potentially subject to error. The simplest approach to the practical problem is to assume that all glassware and other objects coming into contact with the solution become coated with a monomolecular film. All pipettes, flasks etc. should therefore be thoroughly rinsed with the solution before use. With very dilute solutions large volumes should be used in order to avoid any significant change in concentration because of this adsorption.

Obviously the greatest care should be taken in cleaning all glassware and any other equipment before use. Consecutive treatments with soap or Teepol, chromic acid, sodium hydroxide and hydrochloric acid, with very thorough rinsing between treatments and finally, should be adequate (cf. Cousins, 1951).

(ii) *Solubility.* Some otherwise efficient germicides may be limited in usefulness because of their low solubility. An interesting phenomenon that we have encountered is the increased germicidal activities of the slurries which result from the addition of germicides beyond their solubility limits. There are various possible explanations for this, but its importance lies in the fact that with the addition of say 100 p/m of a germicide whose nominal solubility is 10 p/m there may be a significant increase in the disinfectant action over that obtained with 10 p/m. Organic substances of low solubility in water are usually readily soluble in acetone and alcohol, and we have found that an alcoholic solution gives a finer slurry when dilutions are prepared in the usual way.

(iii) *Stability.* All QACs appear to be quite stable in solution, whether heated or held for several years at room temperature (Heineman, 1937; Lawrence, 1950, p. 53; Valko & Dubois, 1945; Mueller *et al.* 1946). Freezing also is without effect (Dunn, 1937; Lawrence, 1950, p. 54).

(iv) *Incompatibilities.* Many anionic compounds neutralize or precipitate QACs and are therefore incompatible in sterilizing formulations (Domagk, 1935; Hornung, 1935; Baker *et al.* 1941*a*,*b*; Valko & Dubois, 1944; Rahn, 1946). Valko & Dubois showed that apparently killed bacteria could be revived by the application of a large molecule anion within 10–30 min. However, not all anionic substances are efficient for this purpose (Lawrence, 1947, 1948). Some incompatibilities are surprising (cf. Lawrence, 1950, p. 25). Thus soluble nitrates may be incompatible with the benzyl QACs because of the low solubilities of the QAC nitrates.

(v) *Associative effects.* The narrow spectrum of some surface active germicides, particularly the QACs, naturally prompts investigations of the possibilities of mixtures of germicides. The results are rarely promising because it is difficult to find two germicides which are complementary in respect of the bacterial genera on which they act. Most disinfectants have either a wide spectrum or similar narrow spectra. It is difficult to find, for example, two germicides both having desirable properties, e.g. absence of odour, corrosive power and toxicity, one of which is particularly effective against Gram-positive bacteria and the other against Gram-negative organisms.

Any harmless substance which enhanced the germicidal power of a QAC would be of great value. Unfortunately some useful concomitants, e.g. non-ionic detergents, may weaken them. It has been claimed that some new types of compounds may enhance the bactericidal power of some QACs, and such a development would be of the greatest interest. The possibility of both synergistic and antagonistic effects should always be kept in mind when testing mixtures.

(vi) *Mixing techniques.* Apart from possible errors in timing in short time tests, e.g. those of 2 min duration, and errors in the transfer of germicide, the method of mixing can affect the result. Thus the addition of a small volume of cell suspension to a large volume of germicide may give a higher kill than the addition of a small volume of germicide to a stabilized cell suspension.

The vigour of shaking can also affect the results by breaking up cell clusters, intensifying the 'washing effect' on cells, and by producing foam. Cousins (1951) found that vigorous shaking often gave a higher percentage kill.

The apparently innocuous procedure of adding an aliquot of a broth culture to a germicide solution and making colony counts at intervals is not so harmless as it appears. We have found that kills up to 50% can be obtained purely by the dilution effect, presumably due to the shock induced by the transfer of the cells to the new environment or to the washing of metabolites away from the cell surface.

(vii) *Contact time.* The periods of contact used in the recognized disinfectant tests are shown in Table 7. Working with QACs, Mallmann & Hanes (1945) used contact times of 1, 5, 10 and 30 min, Berry (1952) used 1 to 480 min and Cousins (1951) used 2 min.

Table 7. *A comparison of the contact periods used in standard tests*

Method	Contact periods (min)						
Rideal-Walker	2·5	5	7·5	10	—	—	—
F.D.A. (U.S.A.)	—	5	—	10	—	15	—
Hygiene Laboratory (U.S.A.)	—	5	7·5	10	12·5	15	—
Chick-Martin	—	—	—	—	—	—	30

In practice a disinfectant should achieve its objective in as short a time as possible. It seems to be generally accepted that a contact time of not less than 2 and not more than 10 min should be used, i.e. there should be a reasonable minimum time for the germicide to act, and a maximum time acceptable in relation to such practical considerations as conveyor throughput, rinsing and staff amenities. It appears to us that these times are reasonable and suitable from all points of view. A possible criticism is that the error of timing for the addition of germicide and the withdrawal of an aliquot must be about 1 sec, and this is about 1% of 2 min. However, the error of the plate count is such that an incidental error of 1% is not important. Where circumstances permit contact to continue for several hours (e.g. overnight), appropriately lengthened times can be used.

The usually accepted formula relating time of reaction, concentration of germicide and number of survivors is $KC^n t = \log(a/b)$, where K is a constant; t, the time; C, the concentration; a, the initial number of organisms; b, the number after time t, and n is the concentration coefficient. However, Jordan & Jacobs (1944, 1945) showed that the logarithmic death rate (i.e. a linear relation between log survivors and time) does not really hold, and that the death rate is rather slow at first, rises to a peak and then declines to an approximately linear value. They related disinfection time to germicide concentration by the formula $C^n t = K$, and were of the opinion that measurements should be based on a high mortality. In their work they were able to determine the 99·999999% mortality time, with survivor levels of about 3 viable cells/ml.

(viii) *Special requirements for skin disinfectants.* The need for a satisfactory disinfectant for skin has been long realized (Senn, 1905). Such a disinfectant must not only be effective against common infective agents; it must be non-irritant and penetrative. It has been claimed that phenylmercuric dinaphthylmethane disulphonate (Penotrane) meets these demands (Reid *et al.* 1958; Goldberg *et al.* 1950). It is said to be adsorbed on to the skin so that its local concentration builds up, and its activity is alleged to be enhanced in the presence of protein material such as blood, serum and pus (Reid, 1959). The latter claim is surprising in view of the fact that all other disinfectants, including mercurochrome (sodium dibromhydroxymercurifluorescein) are less effective in the presence of blood serum. Indeed, this is fundamentally the reason for the failure of the carbolic spray type of treatment (Lister, 1867).

In spite of the low toxicity of surface active germicides, little work has been done *in vivo*. Brewer & McLaughlin (1951) showed that benzalkonium chloride was more effective than other commonly used antiseptics for preventing the onset of tetanus in artificial infection experiments with spores of *Cl. tetani*.

5. THE PHENOL COEFFICIENT TEST WITH QACs

Various explanations have been offered for the anomalies which arise when QACs are tested by the phenol coefficient method, including the following. (i) Unequal distribution of cells due to adsorption (Klarmann & Wright, 1946). (ii) Variations caused by surface tension effects in the volumes of liquids used (Klarmann & Wright, 1946, 1948). (iii) Reactions between constituents of the medium, e.g. phospholipids, and the QAC (Brewer, 1943). (iv) 'Carry over' of QAC in sufficient quantities to exert a bacteriostatic effect. This effect may be reduced if not eliminated by transferring small amounts of the medication liquid to a large excess of growth medium

(Klarmann & Wright, 1946; Resuggan & Davis, 1947). (v) Agglutination of the bacteria and adherence of the charged clumps of cells to the glassware (Klarmann & Wright, 1946). The use of plastic, rubber or paraffin wax coated glassware does not seem to give results different from those obtained with ordinary glassware (Lawrence, 1950, p. 56) and the substitution of pipettes for loops only slightly improved reproducibility (Tice & Pressman, 1945). (vi) The occurrence of foaming. Pressman & Rhodes (1946) mixed the solutions by rotation of the tube and, using a pipette transfer plating method, claimed to have obtained very uniform results. (vii) Persistence of a bacteriostatic effect in the growth medium (cf. section 4, c). To avoid any possibility of this, inactivators may be added to the medium (Armbruster & Ridenour, 1947; Quisno *et al.* 1946; Stuart, 1947). (viii) The appearance of false positives, i.e. the occasional occurrence of growth not in conformity with the general pattern of the results. Various theories have been advanced to explain these (cf. Klarmann & Wright, 1946, 1948), but such results must be due to factors such as the survival of the occasionally occurring highly resistant cell, the agglutination of cells to form a cluster which results in the inner cells not being attacked by the germicide, the protection of a few cells by deposition of media constituents, and/or the agglutination of cells, so that the suspension is no longer uniform and the random sampling error of the loop or pipette becomes very large.

Quite apart from criticisms of the phenol coefficient method which apply specifically to surface active compounds, there are other points to be considered. First, if the test organism is by nature resistant to phenol, a high phenol coefficient may be obtained for a substance which is only moderately effective against that organism. Second, the presence of organic matter, use at other temperatures, and other changes in conditions may give markedly different results if the basic phenol coefficient method is thus adapted, e.g. as in the Chick-Martin test. Phenol coefficients for QACs obtained by the F.D.A. method (Ruehle & Brewer, 1931) vary from about 100 to 500 at 20°, and from about 200 to 1,000 at 37° (Lawrence, 1950, p. 64). Third, as the phenol coefficient method expresses a result in terms of pure phenol, preparations which are solutions of germicides and are tested as such therefore appear less effective than if the active substance(s) were tested. While manufacturers doubtless realize this it does not follow that officials and users generally do.

Modifications of the phenol coefficient method to overcome these disadvantages have been proposed by Barber & Harris (1948), Barber *et al.* (1947), Cade (1947*a*,*b*), James (1947), Eckfeldt & James (1947), Klimeck & Umbreit (1947), McCulloch (1947*a*,*b*), McCulloch *et al.* (1948*a*,*b*), Quisno *et al.* (1947*a*,*b*).

In assessing the true value of QACs as disinfectants it is essential to give consideration to the rather involved technical arguments advanced by workers in this field. It should also be borne in mind that not all workers and authors are free from commercial bias. The crux of the problem seems to be that ever since 1903 many workers in the disinfectant field have failed to realize that the phenol coefficient method was only intended to form a basis for assessing the power of phenolic disinfectants, or of disinfectants acting like phenol on bacteria. These are less affected by organic matter and have a broader bacterial spectrum than certain other types of disinfectant. Moreover, they are more stable chemically than some.

However, as an example of the usefulness of the test when properly understood, one may quote the practical value of using a disinfectant at a dilution of 20 times the Rideal-Walker coefficient. This is based on the fact that 5% phenol is generally recognized as an efficient disinfectant (Stuart *et al.* 1955).

The logical development of this thesis is that while the basic principle of the Rideal-Walker test, which is the use of a chemical yardstick, should be retained, each group of disinfectants should be considered *de novo* both from the point of view of the chemical yardstick and the end point adopted. The most suitable yardstick would appear to be the simplest of the homologous series for the particular group concerned. The end point should be governed by the purpose for which the disinfectant is designed, and especially whether bactericidal or bacteriostatic properties are the prime interest. All conditions such as temperature, time, amount and type of organic matter should be adjusted to be as near to the conditions of use as possible.

Table 8. *Possible reference substances for germicide assessment**

Type of germicide	Reference substance
Phenols	Phenol
Substances giving 'free chlorine', halogens, iodophors	Sodium hypochlorite (determined as available chlorine)
Quaternary ammonium compounds	Cetyltrimethylammonium chloride
Anionic	Sodium stearate
Ampholytic	Dodecyl-β-alanine
Mercurials	Merthiolate
Alkalis	Sodium hydroxide
Acids	Hydrochloric acid
Alcohols	Ethyl alcohol
Aldehydes	Formaldehyde
Gases and vapours	Ethylene oxide

* The stability of some of these suggested reference substances may be a problem (Hirsch & Muras, 1955).

Possible basic reference substances for various types of germicide are given in Table 8. On this basis all germicides would be assessed in comparison with the reference substance under defined conditions of testing.

6. REPRODUCIBILITY

The lack of reproducibility in all tests of QACs, irrespective of the type of test, is well recognized and the question of the number of replicates required is often difficult to answer because of the variety of tests. A simple statistical treatment is possible of the results of tests giving a simple number, e.g. a percentage kill or a limiting dilution, but with tests such as the phenol coefficient, which have wide 'gaps', statistical treatment is more difficult. Lack of reproducibility in colony counts is taken into consideration in the standard formulae for calculating the significance of differences between means, but false positives present a difficult problem. In sorting tests at least three consistent experiments should be made, but making the decision as to when a result is so inconsistent as to be unreliable is difficult. The experienced worker will usually sense an unsatisfactory experiment, but some degree of subjectivity is unavoidable.

It is usually possible to compare two preparations using any one method, and to decide which is superior. Unfortunately another method may reverse this order, and the best procedure then is to base a conclusion on the result given by the method in which the practical conditions of use are most nearly approached.

In all laboratory tests greater accuracy can be expected if the same workers are doing the same tests regularly. The first results by any worker should always be discarded unless they agree with those of subsequent tests. Data on the accuracy of the standard tests have been given by Stuart *et al.* (1958).

We again emphasize that caution should be exercised in the interpretation of any laboratory test of a disinfectant, even if the test is designed to approximate as closely as possible to working conditions. As a guide, we may suggest that if in any laboratory test one disinfectant appears to be at least 10 times as effective as another, then that disinfectant will probably be superior in practice.

7. The Ultimate Objective

All disinfectants and antiseptics are used to prevent the infection of animals or plants, or to prevent the spoilage of food. The ultimate criterion must therefore always be to discover whether the proposed germicide, used as directed, achieves this purpose. No two practical applications of germicides are ever exactly alike, and it is often difficult to standardize all factors under actual working conditions. The value of laboratory tests is threefold: they enable manufacturers and others to make preliminary sorting tests, they permit scientists to compare results from different laboratories, and they enable manufacturers and distributors to quote numerical data in support of their claims.

It will be clear from the evidence which has been adduced on the factors which may affect the efficiency of surface active compounds that the nearer the laboratory test approaches the conditions of use the more valuable will be the test. The quantity assessed should also be the desired objective. For example, if the incidence of infection or the incidence of failures in a food keeping quality test is reduced to nil, it is really of only academic interest if laboratory tests involving inactivation procedures show that pathogenic or food spoilage organisms are surviving in small numbers.

8. Conclusions

Careful study of the available evidence leads to the following conclusions.

(i) No single test is adequate to express either the fundamental or practical value of a disinfectant.

(ii) The QACs vary so much in their properties that it is highly dangerous to generalize about any aspect of their behaviour.

(iii) The nearer the laboratory test approaches the conditions of use, the more reliable the test is likely to be.

(iv) In order to permit estimates of efficiency and to justify claims by manufacturers, one or more standard reference methods should be adopted.

(v) It is unlikely that any one reference method would be acceptable to all manufacturers, testing organizations and users. Each industry or field of interest may require its own reference method, and there should be no objection to this.

(vi) Before accepting a germicide, or a recommended method for using that germicide, use tests should always be made under adverse conditions to ensure that the objective claimed is achieved.

9. RECOMMENDATIONS

It is highly desirable that all purchasers of a disinfectant (using the term in its widest sense) should be given adequate information about its potency. A precise analysis would be useful to chemists, but of little value to others. Moreover, some manufacturers would object to this, and some disinfectants cannot be described in precise chemical terms.

Just as the Labelling of Food Order (Order, 1953) required certain disclosures of composition, and restricts claims for nutritional value, so the Pharmacy and Medicine Act (Act, 1941) requires disclosure of the formula for certain types of disinfectant; but with both foods and disinfectants chemical formulae may mean little to the general public, and skilful advertising can get round the legal restrictions.

If it is accepted that it would be impracticable to require disclosure of formula, and that no one test is adequate to assess the value of a germicide, it would be logical to require all manufacturers to state potencies in terms of at least two tests, and at least two types of organism. It should not be difficult for an official and unbiased organization to prepare short lists of such tests and of suitable type cultures. This would not preclude manufacturers from offering information based on the results of other tests, but it would prevent misleading advertising framed on, say, Rideal-Walker tests where such a test result may mean very little, and claims such as a 99·999% kill which, without specifying the technique used, may mean nothing at all.

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(Symposium on Methods for the Evaluation of the Antibacterial Action of Surface Active Compounds: Paper II)

THE MECHANISM OF THE BACTERICIDAL ACTION OF SURFACE ACTIVE COMPOUNDS: A SUMMARY

By B. A. NEWTON

Medical Research Council Unit for Microbiology, Biochemical Laboratory, University of Cambridge

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1. Introduction

It is now generally accepted that ionic surface active compounds are bactericidal rather than bacteriostatic. Various workers have demonstrated that the proportion of a given bacterial population killed (up to 99%) is related to the amount of the surface active agent added and that maximum killing for a given concentration of surface active compound probably occurs within the first few minutes (Gale & Taylor. 1947; Salton, 1951). Certain substances (e.g. phospholipids, proteins, nucleic acids and soaps) which are known to form complexes with surface active anions and cations will prevent this bactericidal action if added to a bacterial culture before, or at the same time as, the surface active agent, but not after combination of the agent with the cells.

Since the work of Jacobs (1916) and Domagk (1935) aroused interest in surface active compounds as a new group of disinfectants several hypotheses have been advanced to explain their bactericidal action, including a general denaturation of cell proteins, the inactivation of a 'specific detergent sensitive enzyme' and the disorganization of cell permeability barriers. The relative merits of these hypotheses have been discussed in some detail in a recent review (Newton, 1958) and the reader is referred to this for a more complete account of the experimental findings.

2. Evidence for the Disorganization of Cell Permeability Barriers

Briefly, it may be said that the data which have accumulated since the early investigations of Sykes (1939) and Baker, Harrison & Miller (1941) leave little doubt that the treatment of bacteria with certain anionic or cationic surface active compounds results in a very rapid disorganization of a membrane or structure which normally controls the osmotic equilibrium of the cells. Also, a close correlation exists between the bactericidal concentration of a surface active compound and the concentration of that compound which causes an alteration in cell permeability (Salton, 1951), whereas viability is, in general, affected by concentrations lower than those which inhibit metabolism (Sykes, 1939). However, it cannot be said with certainty from these data that the primary action of surface active agents is to disrupt the bacterial cell membrane; the possibility still exists that the observed change in cell permeability is a secondary phenomenon.

The development of techniques for the preparation of bacterial cell walls in a highly purified state (Dawson, 1949; Salton & Horne, 1951) and the demonstration that the protoplast membrane is a definite structural entity of the bacterial cell (Tomcsik & Guex-Holzer, 1952; Weibull, 1953) have permitted a new experimental approach which may ultimately resolve this problem.

From a knowledge of the external conditions required to stabilize bacterial protoplasts (1·0 M sucrose) it can be deduced that the intracellular constituents can exert an osmotic pressure of up to 30 atmospheres across the membrane, which in the intact cell is stabilized mechanically by the cell wall. Thus we can conceive that surface active agents might cause the rupture of the protoplast membrane in two quite distinct ways: they might penetrate the cell wall, which is thought to have an effective pore diameter of *c.* 1 mμ (Mitchell & Moyle, 1956*a*), to combine with and disorganize the underlying membrane or alternatively they might so react with some component of the cell wall that the altered structure would no longer stabilize the membrane, which in a normal environment would rupture spontaneously.

3. INDIRECT DISORGANIZATION THROUGH THE DISSOLUTION OF THE CELL WALL

Evidence that certain surface active compounds do cause the dissolution of bacterial cell walls has come from the work of Salton (1957), who found that purified preparations of cell walls from *Escherichia coli* and *Rhodospirillum rubrum* were rapidly dispersed by the anionic compounds sodium dodecyl sulphate and sodium lauryl sulphate. However, it seems that these compounds also disrupt protoplast membranes, and at the present there is no evidence that any synthetic surface active agent can modify the structure of the bacterial cell wall and leave the protoplast membrane intact. On the other hand the basic polypeptide antibiotic polymyxin has recently been reported so to modify the cell wall of *R. rubrum* that 'protoplast-like' bodies are formed (Tuttle & Gest, 1959); these results differ from those obtained with other organisms (Newton, 1956).

4. DIRECT DISORGANIZATION OF THE PROTOPLAST MEMBRANE

Experimental evidence for the direct action of surface active anionic and cationic substances on the protoplast membrane has been obtained by Tomcsik (1955) and by Newton (1955). When *Bacillus megaterium* was treated with a bactericidal concentration of a detergent followed by lysozyme, the normal transformation to spherical protoplasts did not occur; the protoplast membrane was fixed in a cubical or rod shaped form. Protoplasts from bacteria which had been pretreated with a surface active compound differed from those obtained from untreated cells in that they were no longer sensitive to osmotic shock, suggesting that a marked change in the physico-chemical properties of the protoplast membrane had occurred. Also Gilby & Few (1957), who studied the action of a number of detergents possessing the C_{12} *n*-alkyl chain on *Micrococcus lysodeikticus* and on protoplasts prepared from that organism, found the reactivity sequence for lysis of protoplasts to be the same as that for killing cells, which again suggests that the protoplast membrane may be the site of attack of ionic detergents in intact organisms. This reactivity sequence, which is determined by the ionic groups of the detergents and is as follows: $-NH_3^+ > -N(CH_3)_3^+ > -SO_4^- > -SO_3^-$, is the opposite of that reported for haemolysis by ionic detergents. Pethica & Schulman (1953) previously obtained evidence that cholesterol might be the site of attachment of ionic detergents to the red cell membrane; but Gilby & Few (1957) argued that since a different reactivity sequence is obtained for protoplasts it is unlikely that membrane components of the sterol type, even if present, would constitute the site of attack on protoplasts. These workers detected the presence of phospholipids in the protoplast membrane of *M. lysodeikticus* by infrared spectroscopy and suggested that these would provide suitable ionic groups for electrostatic interaction with both anionic and cationic surface active agents. Further support for this view comes from the observation that bacteria can be protected against the action of certain cationic compounds by divalent metal ions (Valko & DuBois, 1944; Ridenour & Armbruster, 1948; Massart, 1952), and particularly by uranyl ions (Newton, 1954), which are known to bind to phosphate groups (DeJong, 1949; Rothstein & Meier, 1951). However, there is some evidence to suggest that anionic and cationic compounds may not act at the same site on the protoplast membrane. Salton (1957) found that the anionic compound sodium lauryl sulphate completely dispersed protoplast membranes, as judged by turbidity measurements and by phase contrast microscopy, whereas after treatment with the cationic compound cetyltrimethylammonium bromide (CTAB) empty 'fixed' membranes could be seen. These results suggest a fundamental difference in the reaction of these compounds with the protoplast membrane. Similar effects have since been noted by Gilby & Few (1957). The ruptures in the membrane resulting from CTAB treatment are thought to be relatively small, since only substances of low molecular weight are released from the cells; the largest molecule so far observed to leak out of CTAB treated organisms is cytochrome C_3 (Postgate, 1956).

5. UNSOLVED PROBLEMS

Thus there is now evidence that synthetic surface active compounds can combine with, and modify the structure of, two defined components of the bacterial cell, the wall and the membrane. Also it seems that anionic and cationic compounds may have different primary binding sites and may act in quite distinct ways. Clearly the problem of the mode of action of surface active agents is far from being solved and its complete elucidation must await more detailed knowledge of the chemical composition and biochemical function of the protoplast membrane and the bacterial cell wall. The findings of the last four years have shown that the protoplast membrane can no longer be regarded as a relatively inert lipoprotein film which acts mainly as an osmotic barrier; it must be thought of as a dynamic system which actively controls the entry of substrates into the cell (Mitchell & Moyle, 1956*a*: Rickenberg, Cohen, Buttin & Monod. 1956). Also, it may contain a variety of enzymes (Mitchell & Moyle, 1956*b*: Weibull. Beckman & Bergstrom. 1959) and possibly play an important role in protein synthesis (Butler. Crathorn & Hunter, 1958). The disorganization of such an intricate structure by surface active compounds may well prove to be more complex than the simple disruption of lipid monolayers by detergents which can be demonstrated in model systems. The possibility that some surface active compounds may act by inhibiting enzymes located in, and involved in the maintenance of, the membrane rather than disorganizing it by physical means cannot at the present time be eliminated. Goodman & Gilman (1954) summed up the problem in the words 'The mode of action of surface active agents is a highly controversial subject'. This statement would still seem to hold true.

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(Symposium on Methods for the Evaluation of the Antibacterial Activity of Surface Active Compounds: Paper III)

THE ASSESSMENT OF THE ACTIVITIES OF SURFACE ACTIVE AGENTS FOR USE IN THE CATERING INDUSTRY

By BETTY C. HOBBS, N. EMBERLEY, HELEN M. PRYOR
AND MURIEL E. SMITH
Central Public Health Laboratory, London, N.W.9

SUMMARY: The cleaning and bactericidal efficiencies of a number of detergents were estimated by laboratory tests, the techniques of which are described. Field trials with some of the detergents in a small and a large canteen gave results similar to those obtained in the laboratory. The ability of *Salmonella paratyphi* B to survive and grow after inoculation into samples of used dish water obtained from a small canteen was demonstrated.

THE SELECTION of detergents for particular purposes requires the consideration of at least three properties: the efficiency with which a wide variety of food debris is removed, the bactericidal activity of the substance in solution, and the effect of that solution on the hands and on machinery.

The purpose of the work described below was not to enlarge on the merits of each detergent tested but to describe the methods used to test the cleaning power and bactericidal effect of the various substances, grouped according to their composition, and to apply the knowledge so gained to a consideration of their sphere of usefulness in practice.

MATERIALS AND METHODS

Detergents. The detergents studied included, in addition to borax, soap flakes and sodium carbonate. materials which could be placed in the following groups. (a) Mixtures of inorganic salts: these included sodium metasilicate, sodium carbonate, sodium metaphosphate, and other phosphates in varying proportions, and some contained hypochlorite. (b) Household detergents; these included mixtures of anionic organic substances—alkyl benzene sulphonate or secondary alkyl sulphate—with various inorganic salts, e.g. sodium sulphate, sodium silicate, sodium perborate, magnesium sulphate and various phosphates of sodium. (c) Anionic organic substances (e.g. secondary alkyl sulphate) with or without hypochlorite. (d) Cationic organic substances; these comprised various quaternary ammonium compounds either alone or in association with inorganic salts. (e) Nonionic organic substances of which one, based on polyethylene glycol, was tested.

The substances were dissolved in softened London tap water: its hardness, and the pH of the diluted detergent, were determined for each experiment. The dilution for each detergent was the manufacturer's recommendation, when one was given, otherwise 0·3% solutions were used.

Standard soiling mixture. The mixture used was adapted from the formula recommended by Hucker (1942–1943) and reported by Mann & Ruchhoft (1946), and contained: peanut butter, 10 g; butter, 10 g; *national fat, 10 g; flour, 10 g; *dehydrated egg, 10 g; evaporated milk, 15 ml; distilled water, 50 ml; *Mandarin black ink (Windsor & Newton), 4 ml; *Express 44 black ink (Gestetner) diluted 1:1 by weight with boiled linseed oil, 0·5 g; *green micronil, 3·7 ml.

Certain ingredients of the original recipe were unobtainable; the substitutes are indicated by asterisks.

The fats were melted and mixed into a smooth paste. The milk and flour were mixed together to form a homogeneous suspension, combined with the egg and water mixture in a Waring blender at low speed and the Indian ink and printer's ink-oil mixture added. The water and fat mixtures were then beaten together for 5 min, the green micronil added and the mass strained through cheese cloth. Before use 1 part of distilled water was stirred into 5 parts of this 'soil'. The concentration of soil chosen was such that 10±5% was removed by 0·3% borax and 95±5% by 0·3% soap flakes or sodium oleate. The viscosity of this 'soil' was measured. The following materials were unsuitable, because both borax and soap flakes removed them to very similar extents: whole egg; whole egg plus 4% Indian ink; dried milk; water and Indian ink; fat and activated charcoal; and fat plus graphite.

Determination of cleaning efficiency

Use was made of the technique evolved by Mann & Ruchhoft (1946) and modified by Norris & Ruchhoft (1948) and Walker (1948), in which differences in light reading were measured through slides soiled with a standard mixture before and after subjection to detergent solutions in various dilutions and at different temperatures. From the results the percentages of soil removed were calculated.

A replica of the apparatus used by Mann & Ruchhoft (1946) was built. It consisted essentially of a receptacle with an electrically operated reciprocating slide holder supported by a large stand. The difference in the amount of light passing through the soiled slides before and after cleaning was measured by means of a simple photometer designed for the purpose, in which light from a 100 watt bulb passed through the test slides on to a photoelectric cell connected to a galvanometer with a variable resistance in circuit (Fig. 1). The purpose of the resistance was to enable the galvanometer reading given with unsoiled slides in place to be adjusted always to the same value.

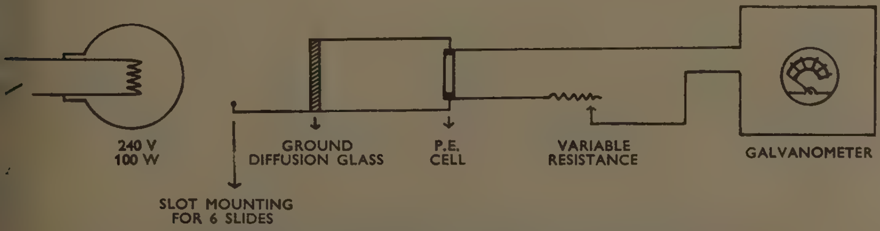

Fig. 1. A diagrammatic representation of the circuit of the photometer used in the estimation of cleaning efficiency.

Glass slides were washed clean and rinsed in hot tap water, boiled in distilled water plus detergent, rinsed in three lots of boiling distilled water, dried on a clean towel and stored in screwcap jars; thereafter they were not touched by hand.

Six slides were used for each test. A light transmission reading (Ic) through these was taken before they were coated with soil, and the galvanometer was set to read 100 by means of the variable resistance. The slides were then placed in racks, dipped momentarily in the soil, drained at an angle of 45° for 3 min, turned and drained for a further 5 min, and finally dried at 95° for 1 hr before being stored overnight in a desiccator.

A second light reading (Is) was obtained for the same set of soiled slides. They were then placed in the slide holder of the washing machine and the washing jar, containing approximately 2 l. of diluted detergent at 60° (or other desired temperature), was raised to immerse them. They were then washed for exactly 3 min (a stopwatch was used), with continual agitation, after which the detergent receptacle was lowered and the excess solution removed by running the machine for 5–6 min with an empty container in place of the washing jar. The detergent solution was then replaced by hot tap water at 80°, the slides were rinsed for 2 min, and the excess rinse water removed in a similar way. The slides were removed and allowed to dry in the air for 15–20 min at room temperature; after this a third light reading (Iw) was taken.

The percentage of the soil removed (R) was calculated, using the formula:—

$$R=\frac{100\,(\log Iw-\log Is)}{\log Ic-\log Is},$$

where Iw is the reading with 6 washed slides, Is is the reading with 6 soiled slides, and Ic is the reading with 6 clean slides (=100). In all cases four runs were done, using slides soiled on different days, and the mean and its standard deviation were calculated. Although the results have been calculated to three significant figures, this probably gives a false impression of the accuracy of the method as the original readings could not be made to more than one significant figure.

Determination of bactericidal efficiency

Laboratory tests. Quantities of 4·5 ml of 10^{-1}–10^{-6} dilutions in saline of each detergent were inoculated with 0·04 ml of a suspension of each of the test organisms in Ringer's solution to give a final density of *c.* 10^4 viable organisms/ml, though in some trials a heavy inoculum of 5×10^6–5×10^7 viable organisms/ml was also used. The test organisms were *Escherichia coli* I and a coagulase positive staphylococcus, phage type 6/47/53.

The suspensions were added to the detergent dilutions at 30 sec intervals by means of a dropping pipette delivering 0·02 ml/drop and a standard loopful (5 mm diameter) of suspension from each tube was transferred to nutrient broth after 10 min contact. The broth cultures were incubated at 37° for 48 hr, examined for growth and plated on either nutrient agar or MacConkey's agar for purity.

Field trials: dishwashing by hand in a small restaurant. The bactericidal effect of various detergents was investigated in the kitchen of a small restaurant where the normal procedures for washing crockery by hand were in use. The presumptive coli-aerogenes content (Report, 1956) of wash water containing the recommended concentration of detergent was estimated before and after a fixed number of soiled plates had been washed in it. As the initial counts were low, the probable number of coli-aerogenes bacteria could be used as an index of the relative bactericidal action. The temperature and pH of the water were determined at the beginning and end of the experimental period. As another measure of relative action, swabs were taken from 5 of each batch of washed plates, using the technique described by Higgins & Hobbs (1950) and Higgins (1950*a*,*b*), while the colony count of the wash water before and after use was also determined by the method of Miles & Misra (1938).

Field trials: the washing of pots and pans in a large canteen. Although the use of sterilizing temperatures for rinse waters is common practice for crockery and cutlery, few attempts have so far been made to improve the washing-up conditions for cooking vessels and utensils. Samples of both wash and rinse waters have frequently contained >1,800 faecal coli/100 ml. The effectiveness of various bactericidal detergents for pan washing was investigated in a large canteen, where a few detergents, mostly advertised as possessing bactericidal properties, were recently tested in the wash water of a large double sink unit used for pots and pans.

The ability of Salmonella paratyphi *B to grow in used dish water.* Samples of dish water, which for the purpose of this experiment contained no detergent, or soda only, were collected from the sink of a small canteen (catering for 150 people) after most of the crockery and cutlery used for the midday meal had been washed. The temperature was observed immediately, a note was made of the foods which had been eaten, the opacity was determined by comparison with Wellcome opacity tubes (turbidity range 1–10) and the pH was obtained electrometrically. Surface colony counts were then made on blood agar at 37° and 22° and an estimate of the coli-aerogenes content was obtained by inoculating from the 10^{-1} and 10^{-2} dilutions into MacConkey's broth, incubating at 37° and subculturing positive tubes into brilliant green-bile broth and peptone water at 44°.

The samples were then inoculated with a diluted overnight culture of *Salm. paratyphi* B, and control tubes of quarter-strength Ringer's solution or tap water (and also nutrient broth) were inoculated with a similar quantity of culture. After 0, ½, ¾, 1 and 2 hr at room temperature, and after 18 hr at 37°, surface colony counts of the organism were made on deoxycholate-citrate agar and MacConkey's agar.

Experiments and Results

The experimental work comprised laboratory tests of the various detergents for cleaning power and bactericidal effect, practical field tests in small and large canteens, and an investigation of the ability of *Salm. paratyphi* B to grow in samples of used dish water from a small canteen.

The efficiency of 'soil' removal

A summary of the results is given in Table 1. The cleaning powers of the inorganic mixtures and household detergents were good, but indifferent results were obtained with the detergents in the other three groups. The greatest variation in the standard deviations was obtained with the poorest cleaning agents. The household detergents were rated evenly with soap, and they are approximately the same price; some were more alkaline than soap, but they have the advantage of not precipitating in hard water. A series of tests in hard water might show them to be more effective than soda; they may also be as effective when more dilute. Washing soda, which is cheap, gave surprisingly good results.

Table 1. *A comparison of the cleaning efficiencies of various detergents*

Detergent	Concn. (% w/v)	pH	Hardness of water (degrees)	Extent of soil removal (range, %)	Range of standard deviation
Borax	0·3	10·0	8·0–11·1	8·6–16·7	(±0·94 – ±6·33)
Soap flakes	0·3	9·0	8·8–14·0	97·8–99·1	(±0·23 – ±1·05)
Sodium carbonate	0·3	11·0	9·1	85·6	(±9·02)
Household powders	0·3	7·5–11·0	8·0	95·3–98·5	(±0·87 – ±3·43)
Inorganic mixtures	0·3	>11·0	9·0	85·6–91·8	(±4·81 – ±9·02)
Anionic mixture	0·3 1·0	7·0	9·0–10·1 8·5– 9·0	<1–45·1 56·3–94·3	(±4·12 – ±36·6)
Cationic mixture	0·6–1·0	8·0–11·0	7·5–11·0	<1–17·8	(±19·6)
Nonionic mixture	0·3–0·5	7·0	9·0– 9·1	<1–11·0	(±28·9)

The anionic detergents, considering their price and popularity, gave poor results in the dilutions recommended; stronger solutions were more satisfactory. The cationic compounds were poor detergents in the concentrations tested, but great cleaning power has never been claimed for them and their cost prohibits their general use in higher concentrations; they are claimed to have greater value for bactericidal purposes. The nonionic products also gave a poor performance.

Undoubtedly the organic compounds need to be mixed with inorganic substances for the best results to be obtained.

Bactericidal efficiency

Laboratory tests. A summary of the results is given in Table 2. The inorganic detergents killed the *E. coli* in the 10^{-2} dilution, whereas the staphylococci were sometimes killed only at a dilution of 10^{-1} or not at all; with the addition of chlorine certain of the inorganic detergents were bactericidal in the 10^{-4} and 10^{-3} dilutions respectively. The household powders were effective against the staphylococci at 10^{-2} or 10^{-3}, but had little power against the *E. coli* culture. An anionic detergent was active against the staphylococci in a dilution of *c.* 2×10^{-3} but at 10^{-1} only against the *E. coli*; the (advertised) presence of chlorine in another anionic detergent merely raised the bactericidal power against *E. coli* (to 4×10^{-3}), there being only the same level of activity against the staphylococci.

The cationic substances, either alone or with other materials, were usually effective at dilutions of 10^{-3} to 10^{-5} and in most instances their actions on both organisms were similar.

Nonionic detergents mixed with cationic substances showed a variation from no effect on *E. coli* at 10^{-1} to effectiveness at 10^{-3} against the staphylococci.

It was concluded that the laboratory results did not always substantiate the claims of the manufacturer.

Table 2. *A comparison of the bactericidal efficiencies of various detergents by a laboratory test*

Detergent	Concn. (% w/v)	pH	Lethal dilution for:	
			Staph.	*E. coli*
Borax	0·3	10·0	S*	S
Soap flakes	0·3	9·0	S	S
Sodium carbonate	0·3	11·0	S	10^{-1}
Inorganic mixtures	0·3–1·2	9·0–11·0	10^{-1}–10^{-2}	10^{-2}
Inorganic mixtures plus chlorine	0·6–1·0	9·0–11·0	10^{-3}	10^{-4}
Household powders	0·3–0·5	7·0–11·0	10^{-2}–10^{-3}	10^{-1}
Anionic	0·3	7·0	1·7 × 10^{-3}	10^{-1}
Anionic plus chlorine	0·3	6·0	2·0 × 10^{-3}	4 × 10^{-3}
Cationic	0·15–1·0	7·0–9·0	10^{-3}–10^{-5}	10^{-3}–10^{-4}
Cationic plus inorganic	0·15–0·6	7·0–9·0	10^{-3}–10^{-5}	10^{-2}–10^{-5}
Cationic plus inorganic plus anionic	0·6	9·5	10^{-3}	10^{-3}
Nonionic	0·5	7·0	S	S
Nonionic plus cationic	0·3–0·6	7·0	10^{-2}–10^{-3}	10^{-1}–10^{-2}

* S: the organisms survived exposure to the 10^{-1} dilution, which was the strongest solution tested.

Table 3. *A comparison of the abilities of various detergents to limit the numbers of bacteria in water used for washing plates by hand in a small canteen*

Detergent	pH of water	Counts in the dish water†				Colony count on the washed plates‡
		Coli-aerogenes (MPN/100 ml)		Colony count (/ml at 37°)		
		Before use	After use	Before use	After use	
Borax	9·5	5*	>1,800* 350**	5,000	8,200	900
Sodium carbonate	10·0	<1	>1,800*	43,000	2,700	1,300
Inorganic mixtures	10·0–11·0	<1–7* 4**	<1–>1,800*	<50–2,300	500–7,300	500–12,000
Inorganic mixtures plus chlorine	10·0–11·0	<1	<1	<50	<50–1,300	<25
Household detergents	7·5–<11·0	<1–1,600*	<1–>1,800* 8**	<50–50	<50–6,500	<25–400
Anionic	7·5	8*	>1,800*	<50	350	<25
Anionic plus chlorine	9·0	<1	250*	<50	1,800	200
Cationic plus inorganic	10·0	13*	600*	<50	1,100	500
Cationic plus nonionic	7·0	<1	>1,800* 8**	<50	<50	600
Cationic plus inorganic plus nonionic	8·0	<1	600*	<50	150	<25

* Non-faecal. ** Faecal. † Temperature: before use, 110° F; after use 94–100° F.
‡ Swab method, colonies/plate at 37°.

Field trials. The results of the experiments to determine the bactericidal activities of the detergents under practical conditions of use in a small restaurant where dishes were being washed by hand are shown in Table 3.

Some of them resemble those obtained in the laboratory and indicate that inorganic mixtures and household detergents with and without the addition of hypochlorite may prejudice the survival of coli-aerogenes bacteria.

The cationic substances were not so effective in this field trial as had been expected from the laboratory tests.

The results of the tests of a few (advertised) bactericidal detergents in the washing of pots and pans in a large canteen also indicated that it was possible to repress the coli-aerogenes bacteria in wash water. Further, provided the rinse water was maintained at a reasonable temperature, there would be no further build up of such organisms.

The growth of Salm. paratyphi *B in used dish water*

Table 4 gives the results of the counts of *Salm. paratyphi* B after 0, 2 and 18 hr in dish water (E–M) and from the control suspensions in quarter-strength Ringer's solution (E–H) and tap water (I–M). In addition, the opacities of the uninoculated samples and their surface colony counts on blood agar at 37° and 22° are given, with

Table 4. *The ability of* Salm. paratyphi *B to survive and grow in various samples of used dish water*

Sample	Opacity	Relevant meal	Colony count† (/ml) at		Time after inoc. (hr)	Colony count of *Salm. paratyphi* B (/ml) in	
			37°	22°		Dishwater	Control*
E	1·5	Meat pie and cabbage, fruit salad and custard	$1\cdot4\times10^{6}$	$1\cdot3\times10^{5}$	0	$9\cdot9\times10^{5}$	$8\cdot8\times10^{5}$
					2	$6\cdot8\times10^{5}$	$1\cdot8\times10^{4}$
					18	$6\cdot6\times10^{7}$	<500
F	9·5	Egg and chips, mince pie and custard	$3\cdot6\times10^{5}$	$2\cdot1\times10^{4}$	0	$7\cdot3\times10^{5}$	$7\cdot1\times10^{5}$
					2	$5\cdot8\times10^{5}$	$5\cdot4\times10^{5}$
					18	$9\cdot1\times10^{7}$	$7\cdot9\times10^{3}$
G	7·5	Bacon, peas, gravy and potatoes, treacle tart	$9\cdot1\times10^{5}$	6×10^{3}	0	$7\cdot7\times10^{5}$	$4\cdot7\times10^{5}$
					2	$4\cdot7\times10^{5}$	$1\cdot2\times10^{5}$
					18	$3\cdot6\times10^{5}$	$8\cdot8\times10^{2}$
H	1·5	Stew, mashed potatoes and Brussels sprouts, apple pie and custard	$1\cdot5\times10^{4}$	$1\cdot9\times10^{2}$	0	$1\cdot4\times10^{6}$	$1\cdot3\times10^{6}$
					2	$1\cdot0\times10^{6}$	$1\cdot1\times10^{6}$
					18	$1\cdot5\times10^{8}$	$1\cdot3\times10^{6}$
I	4·5	Egg and chips, marmalade tart	$2\cdot4\times10^{4}$	Not done	0	$1\cdot3\times10^{6}$	$1\cdot2\times10^{6}$
					2	$1\cdot2\times10^{6}$	$<5\times10^{3}$
					18	$2\cdot2\times10^{8}$	<50
J	6·5	Spam, beef, gravy and potatoes, and treacle tart	$3\cdot0\times10^{4}$	$2\cdot9\times10^{4}$	0	$4\cdot9\times10^{4}$	$2\cdot4\times10^{4}$
					2	$4\cdot8\times10^{4}$	$0\cdot3\times10^{4}$
					18	$2\cdot6\times10^{6}$	<50
K	3·5	Meat pie, cabbage and potatoes, baked custard	$3\cdot8\times10^{3}$	$2\cdot3\times10^{3}$	0	$2\cdot8\times10^{3}$	$2\cdot5\times10^{2}$
					2	$2\cdot2\times10^{3}$	$<5\times10^{2}$
					18	$9\cdot9\times10^{8}$	<50
M	4·5	Roast beef, potatoes and cabbage, gravy, mince tart and custard	$2\cdot9\times10^{5}$	$3\cdot6\times10^{5}$	0	$9\cdot9\times10^{4}$	$1\cdot1\times10^{5}$
					2	$8\cdot2\times10^{4}$	<50
					18	$3\cdot9\times10^{7}$	<50

* Samples E–H in Ringer's solution; samples I–M in tap water. † On blood agar.

the nature of the relevant meal. The temperature varied from 43–53° and the pH from 7·1–8·2.

The numbers of *Salm. paratyphi* B dropped a little during the first 2 hr at room temperature, but after overnight incubation at 37° there was usually a marked rise, indicating that in most instances (sample G is an exception) the wash water, which contained no detergent or soda only, not only permitted survival but encouraged growth. The control suspensions, more especially those in tap water, showed a pronounced reduction in numbers, but there was one exception, sample H. In the broth control tubes the organism grew according to the usual pattern.

The results indicate that *Salm. paratyphi* B is able to grow in dish water containing debris from many different types of food. The organisms would be transferred to the rinse water, and unless this was maintained at the recommended temperature of 170–180° F they would be passed on to hands, draining racks and cloths; furthermore wet plates stacked in a pile could retain organisms from day to day.

Discussion

The method used for estimating cleaning efficiency was practicable and easy to perform, although simpler tests have been described for the visual assessment of cleanliness in the field, e.g. the application to washed plates of a dilute solution of carbol fuchsin or iodine (Norwegian Regulations) or of powdered carbon (Brookes & Fennell, 1952). The results obtained were good for soap, sodium carbonate and the inorganic mixtures, and unexpectedly poor for many of the organic substances. It is suggested that the hardness of the water is a significant factor in the estimation of detergent performance. In the washing of dishes by hand the best results would be obtained with mixtures of organic and inorganic materials adjusted to a pH between 7 and 8.

The laboratory method used for determining bactericidal activity was simple and standardized as far as possible, and it appeared to be adequate for comparative tests. It will be seen from Table 2 that the cationic substances were the most efficient, although the combinations of inorganic components with hypochlorite were also good.

The field tests carried out in a small one-sink establishment indicated the sphere of usefulness of the various detergents tested and the results were similar to those obtained in the laboratory. Recent experiments with large sinks for pot washing have shown that an efficient bactericidal detergent will successfully inhibit coli-aerogenes bacteria in the wash water and that, provided this is followed by a hot water rinse, they will not reappear. It is suggested that, in the absence of really hot rinse water thermostatically controlled at a certain temperature, a bactericidal agent should also be added to the rinse water.

Recommendations for dish washing by hand include the use of two or three sinks when a machine is not available. One sink holds the wash water containing detergent, another should be able to maintain water at a temperature of 170–180° F, both to rinse and to sterilize and a third would provide facilities for a hand operated spray following the removal of gross debris before washing.

Unfortunately there are still kitchens where not more than one sink is used and under these conditions a bactericidal detergent would be helpful.

The demonstration of the survival and growth of *Salm. paratyphi* B in dish water samples emphasizes the potential danger of careless washing-up.

The replica of the apparatus originally designed by Mann & Ruchhoft (1946) was made by Mr. W. F. Emberley. The authors are grateful to Mr. T. Nash for designing and making the photometer and for help in other ways. The co-operation of the many manufacturers who sent samples of their products and who willingly provided information about the ingredients is also gratefully acknowledged.

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THE EVALUATION OF SURFACE ACTIVE DISINFECTANTS FOR USE IN MILK PRODUCTION

By CHRISTINA M. COUSINS, W. A. HOY and L. F. L. CLEGG

National Institute for Research in Dairying,
University of Reading

SUMMARY: Details are given of a can test for evaluating detergent-sterilizers intended for farm dairy use. The materials are compared with a standard hypochlorite-detergent solution for their ability to cleanse milk-soiled metal surfaces resembling those encountered in practice.

Results from the can test have been compared with those from a suspension test. This comparison suggests that the suspension test is of value for the initial screening of QAC-detergent mixtures, since those with poor disinfectant properties in suspension acted poorly on surfaces. However, products giving good disinfection in suspension may fail to cleanse a soiled surface if they are poor detergents.

The problem of assessing chemical disinfectants for dairy purposes has been accentuated by the development of materials intended for cleaning and disinfecting in one operation. Clegg (1955) discussed the principles underlying laboratory and field tests for disinfectants intended for farm dairy use and described tests then being developed at the National Institute for Research in Dairying. He concluded that for some years the field trial would probably have to be regarded as the final test before approval could be given to new materials. At that time sodium hypochlorite was the only chemical approved as an alternative to steam or boiling water for cleansing dairy utensils under the Milk & Dairies Regulations (1949).

In 1957 the first products containing quaternary ammonium compounds (QACs) were approved after extensive field trials in which they were compared with hypochlorite used in combination with a detergent (Clegg & Hoy, 1957). In addition the materials were subjected to the Hoy Can Test (Hoy & Clegg, 1953; Clegg, 1955) and from the results it was considered possible to rely on this test to evaluate new formulations (Clegg, Hoy & Cousins, 1959). It has seemed desirable to describe in detail this method which is now the basic test for the approval of detergent-sterilizers (mixtures or formulations of detergents and disinfectants) based on QACs, chlorine or iodine, for use in England and Wales.

Since 1955 statistical design of experiments and analysis of results have made possible a study of conditions affecting the precision of the test. The method of soiling, treating and rinsing individual cans has changed very little; the experimental design (i.e. the arrangement of cans, the order of treatment, the number of materials examined concurrently and the replication) is, however, the outcome of much experimental work. In common with all disinfectant tests successful application depends on meticulous preparation and attention to detail at every stage.

The Can Test is not suitable for general use as a screening test because it is cumbersome and time consuming and requires special facilities and equipment. A comparatively simple suspension test (substantially similar to that described by Cousins, 1951) has therefore been applied to a number of detergent-sterilizers containing QACs and the results compared with those obtained using a can test.

THE HOY CAN TEST

Materials and Methods

Where detergent-sterilizers intended for cleaning and sterilizing (i.e. cleansing) in one operation are to be evaluated, each material is tested at the concentration proposed by the manufacturer (the use-concentration) against a standard hypochlorite-detergent solution (300 p/m available chlorine, 0·25% Na_2CO_3) corresponding to the use-dilution recommended by the Ministry of Agriculture, Fisheries & Food (1959) for cleansing farm dairy utensils. In addition, tests with all solutions at ⅔ use-concentration are included to detect materials which, on dilution, show a decline in efficiency greater than that shown by hypochlorite. Such products might cause difficulties in practice where solutions are not always prepared by accurate measurement.

Equipment. Testing is done in a room about 12 ft^2 in area with a well-drained impervious floor, and equipped with a supply of hot and cold water, steam, a fan, a thermostatically controlled heating system capable of maintaining the room at 22–24°, a steaming block for cans, a wash trough, thermometers, measures, buckets, brushes, etc. Some specially designed equipment is also required, consisting of a rack for stacking the cans (Plate 1, a), a can roller and a handle which clips to the base of the can (Plate 1, b), 12 perforated zinc lids to fit the cans and 2 long handled rubber squeegees (Plate 1, c).

The test surface. Tinned steel milk cans in good physical condition provide, in a convenient form, a surface common to many farm dairy utensils. Five-gal cans have replaced the 10-gal size used originally because the smaller cans are more easily handled. New cans are prepared for use by scrubbing with warm (44–46°) detergent solution (0·25% Na_2CO_3, 0·05% sodium sulphite), rinsing and steaming for 3 min. This treatment does not completely remove the greasy 'bloom' initially present on the surface of new cans: therefore, when the cans are cool, ¼ pt of raw milk is put into each and the cans rolled once daily for 5 days to wet the inner surfaces with milk. When the milk has soured, usually within 2 days, it is replaced with fresh milk. The cans are then washed and steam sterilized as before. Unless the surfaces are 'conditioned' in this way, milk will not form an unbroken film on them.

Washing, rinsing, steaming and drying are repeated after each test run, and if the cans are not used within 24 hr they are steamed again before use. The 12 cans used are permanently numbered.

Milk for soiling the cans. For each test, raw milk (3 pt) from a bulk of at least 1,000 gal is incubated at 22° until it has a methylene blue reduction time at 37° of

15–30 min, but it must not clot on boiling. If not used immediately, the milk is cooled and refrigerated. Just before use, 1 pt of tap water is added and the milk strained to remove any particles of churned fat. A sample is plated on Yeastrel milk agar (YMA) and incubated for 3 days at 30°. The colony count is usually between $2\text{–}20 \times 10^6$/ml.

Soiling the cans. The soiling and disinfectant treatment of each can is shown diagrammatically in Fig. 1. The first can is rolled to spread the milk completely over the inner surface, excluding the neck and shoulder, and is then placed upright, until all the cans have been so treated, when the rolling is repeated. Each can is inverted to drain, and then stacked on the rack; finally the lids (Plate 1,c) are placed over the necks of the inverted cans to reduce air movement in them (Plate 1,a), and they are left overnight (16–17 hr) in the closed room at 22–24°. Not more than 3 ml of milk remains in the can after drainage. The following morning the inner surfaces of the neck, shoulder and bottom of the cans are wiped with a sterilized cloth moistened in hypochlorite solution (300 p/m) to remove visible traces of milk film, since these areas are not included in the test surface.

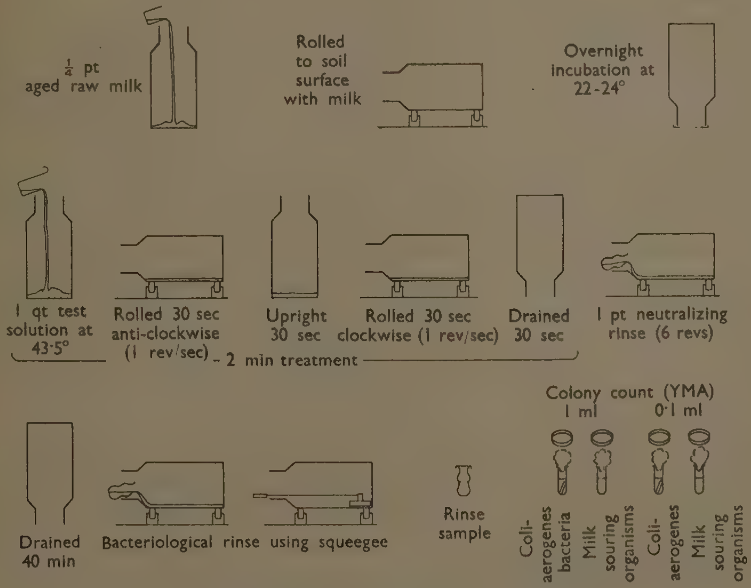
$\frac{1}{4}$ pt aged raw milk
Rolled to soil surface with milk
Overnight incubation at 22-24°
I qt test solution at 43·5°
Rolled 30 sec anti-clockwise (I rev/sec)
Upright 30 sec
Rolled 30 sec clockwise (I rev/sec)
Drained 30 sec
I pt neutralizing rinse (6 revs)
2 min treatment
Colony count (YMA)
I ml
0·I ml
Drained 40 min
Bacteriological rinse using squeegee
Rinse sample
Coli-aerogenes bacteria
Milk souring organisms
Coli-aerogenes
Milk souring organisms

Fig. 1. Diagrammatic representation of the Can Test.

Preparation of test solutions. The appropriate quantity of test material is added to 1 gal of tap water at about 46° in a bucket. Usually 6 materials are tested concurrently and about 5 min before treating the first can all 6 solutions are prepared, in the order in which they are to be used, in buckets numbered 1–6, which are placed in water at about 46° to prevent cooling. When 6 cans have been treated, solution 1 is diluted to $\frac{2}{3}$ use-concentration by adding 1 qt of tap water to 2 qt of solution, and the other 5 solutions are similarly diluted.

Disinfectant treatment. One quart of test solution at 43·5° is poured into the upright can (avoiding the sides) which is then placed on the roller (Plate 1,b) and treated as shown in Fig. 1. During the first 30 sec of the 2 min treatment, a small squeegee is applied to the neck and shoulder of the can to bring the solution in contact with these surfaces and to remove any remaining film. During the next 30 sec the squeegee is applied to the bottom of the can. After exactly 2 min, 1 pt of sterile water containing the appropriate neutralizer is added to the can which is revolved 6 times, inverted to discard the neutralizer and drained on the rack. All the cans are similarly treated in order 1–12.

Neutralizer for disinfectants. For products containing available chlorine or iodine, 10 ml of sterile 7% $Na_2S_2O_3.5H_2O$ are added to each pint of sterile water whereas for QACs 10 ml of a sterile solution containing 3% of Lubrol W (Imperial Chemical Industries, Ltd.) and 2% of egg lecithin are used.

Bacteriological examination of the treated cans. After each can has drained for 40–50 min, 250 ml of quarter-strength Ringer's solution are added. The can is then rolled 12 times and placed upright for about 5 min. The can is rolled again and at the same time the rinse is applied systematically with a sterile long handled rubber squeegee (Plate 1,c), the surface of the body of the can being squeegeed 3 times. This treatment is more effective than rinsing alone in removing residual film and bacteria. The rinse is sampled for bacteriological examination.

Control can. An indication of the initial level of contamination in the cans is obtained by rinsing a soiled, untreated can. This control count, which varies between 10^8–10^9 bacteria/can, is not required for assessment of the efficiency of test materials.

Bacteriological and chemical tests. Within 2 hr of sampling the can rinses are tested for (a) colony count on YMA (duplicate plates), (b) dilution count of coli-aerogenes organisms in MacConkey's broth, and (c) dilution count of milk souring organisms (MSO) in litmus milk. Incubation is for 3 days at 30°. Only the colony counts are used in the statistical analysis of the results, the other counts serving to indicate whether a product is selective in its killing action.

A sample of each test solution taken just before use is tested for disinfectant content to ensure that the correct concentrations have been used. Chlorine and iodine preparations are titrated with standard sodium thiosulphate solution, and QACs are estimated by the method of Chinnick & Lincoln (1954).

Duration of can test procedure. Two experienced workers are required to apply the carefully timed routine as follows:—

Soiling the cans	15 min
Preparation of soiled cans and of the test solutions	35 min
Disinfectant treatment and neutralizing rinse for 12 cans	32 min
Bacteriological rinsing	18 min.

Washing up and sterilizing cans and equipment takes about an hour.

Experimental design. Usually 5 materials and the standard hypochlorite preparation are examined concurrently and on each of 6 different days, using 12 cans each day. Thus, on any one day, each material, including hypochlorite, is tested on 2 cans, one with the material at use-concentration and the other with the material at $\frac{2}{3}$ use-concentration. The allocation of materials to cans conforms to a 6×6 Latin square to overcome effects due to differences between individual cans, position of cans during incubation, and order of testing. An example of the design for a series in which 5 materials (A, B, C, D & E) were compared with hypochlorite (H) is shown in Table 1. At each stage of the test the permanently numbered cans are treated in order 1–12.

Table 1. *Experimental plan for testing* 6 *materials on* 6 *days*

Day	Cans tested with use-concentration, nos					
	1	2	3	4	5	6
	Cans tested with $\frac{2}{3}$ use-concentration, nos					
	7	8	9	10	11	12
1	H	C	B	D	A	E
2	D	B	A	E	H	C
3	A	E	D	B	C	H
4	B	D	H	C	E	A
5	E	A	C	H	B	D
6	C	H	E	A	D	B

Analysis of results. Analyses of variance are carried out on the logarithmic transformations, $y=\log(x+1)$, of the original colony counts/ml of rinse. The differences between the mean values of the transformed counts for the test materials and the mean value for the standard hypochlorite detergent solution are tested for significance. Where the mean value for a material is greater than that for hypochlorite and the difference is significant at the 5% level at least, for one or both concentrations, the material fails the test. Products giving results as good as hypochlorite, or better, are considered to have passed the test.

RESULTS

An example of results obtained using 5-gal cans is given in Table 2. In this series none of the materials (A–E) failed the test and one (D) was better than hypochlorite (H) at both concentrations. Two more sets of results from earlier experiments using 10-gal cans, exemplifying extremes in colony count given by the standard hypochlorite solution in different series, are shown in Table 3. From the data in the Tables, materials giving results significantly better or worse than hypochlorite at the different probability levels can be distinguished by inspection of the derived mean colony counts (corrected for bias according to Quenouille, 1950).

Factors affecting the level of results. Some day-to-day variation in the number of survivors in treated cans is to be expected because the flora of the milk varies daily, and because of minor fluctuations of temperature and humidity during incubation of the soiled cans. This variation might seriously affect the results if absolute

Table 2. *Transformed colony counts, log (colony count/ml+1), for rinses of 5-gal cans treated with detergent-sterilizer solutions for 2 min at 43·5°*

Test material	Use-concentration mean	⅔ Use-concentration mean
A	1·01	1·64
B	0·52	2·03
C	0·83	1·55
D	0·73	1·01
E	0·62	2·03
Hypochlorite (H)	1·49	1·93

Differences between means		SE (20 d.f.)	Differences between means	SE (20 d.f.)
A–H	−0·48	0·31	−0·29	0·27
B–H	−0·97**		0·10	
C–H	−0·66*		−0·38	
D–H	−0·76*		−0·92**	
E–H	−0·87*		0·10	

Test material	Mean colony count/ml of rinse (derived back from transformed values)	
A	20·6	77·2
B	6·07	193
C	13·2	62·5
D	10·4	17·4
E	7·86	193
Hypochlorite (H)	64·1	152

* 0·05>P>0·01 ** 0·01>P>0·001

Table 3. *Transformed colony counts, log (colony count/ml+1), for rinses of 10-gal cans treated with detergent-sterilizer solutions for 2 min at 43·5°*

Experiment A			Experiment B		
Test material	Use-concn. mean	⅔ Use-concn. mean	Test material	Use-concn. mean	⅔ Use-concn. mean
F	0·00	0·92	M	0·94	1·74
G	0·58	1·37	N	1·33	2·30
J	1·95	3·66	O	0·90	1·46
K	2·75	3·96	P	1·84	3·24
L	0·08	1·07	Q	3·13	4·50
Hypochlorite	0·31	0·50	Hypochlorite	2·22	2·76
Differences between means			Differences between means		
F–H	−0·31	0·42	M–H	−1·28**	−1·02**
G–H	0·27	0·87*	N–H	−0·89*	−0·46
J–H	1·64***	3·16***	O–H	−1·32**	−1·30***
K–H	2·44***	3·46***	P–H	−0·38	0·48
L–H	−0·23	0·57	Q–H	0·91*	1·74***
SE (20 d.f.)	0·31	0·41	SE (20 d.f.)	0·34	0·32
Mean colony counts/ml of rinse (derived back from transformed values)			Mean colony counts/ml of rinse (derived back from transformed values)		
F	1·14	31·0	M	21·2	126
G	7·18	90·5	N	53·4	453
J	188	17,800	O	19·0	65·4
K	1,220	35,400	P	175	4,030
L	1·58	44·5	Q	3,470	72,500
Hypochlorite	3·38	11·3	Hypochlorite	421	1,310

* 0·05>P>0·01 ** 0·01>P>0·001 *** P<0·001

standards were imposed. However, a product is not required to produce bacteriologically satisfactory cans since the final result depends on a comparison with hypochlorite.

For good discrimination it is desirable that hypochlorite shall not give sterile cans, but with 300 p/m available chlorine this is sometimes unavoidable and is one reason why all the materials are tested at $\frac{2}{3}$ use-concentration. The relative humidity (RH) of the atmosphere during overnight incubation is important. If the milk film in the cans dries too quickly it seems that the bacterial population fails to increase or some bacteria die during overnight incubation, and the colony counts of many of the treated cans may be too low for reliable estimates. A suitable RH must be determined by experiment because it will vary according to the rate of air movement needed to maintain an even temperature around the cans. In a room with a powerful fan an RH of 70–75% will be necessary; with slow air movement 50% may be adequate.

The Can Test as a preliminary screening test. Full evaluation of a test material may take 3 weeks or more. However, useful information about an experimental formulation, for example, can frequently be obtained from a one-day test. Usually one concentration only, tested on 6 cans, is compared with standard hypochlorite-detergent also tested on 6 cans. With a markedly inferior material the results are clear-cut and full scale tests are unnecessary. An illustration is given in Table 4.

Table 4. *A QAC-detergent 'screened' by a one-day Can Test*

Test solution	Can no.	Colony count/ml of rinse	Coli-aerogenes bacteria /ml of rinse	MSO* /ml of rinse
QAC-detergent $\frac{2}{3}$ use-concn.	2	3,870	+	+
	4	3,300	+	+
	6	10,300	+	+
	8	3,875	+	−
	10	2,310	+	+
	12	3,600	+	—
Hypochlorite-detergent (200 p/m av. Cl $+0{\cdot}17\%$ Na_2CO_3)	1	11	+	−
	3	11	+	−
	5	17·5	+	−
	7	46	−	−
	9	19	−	−
	11	10	+	−

* Milk souring organisms.

THE SUSPENSION TEST

Materials and Methods

Fourteen proprietary products all containing one or more QACs mixed with detergents of different types were examined. Details of analysis were not available for all products, but each manufacturer had recommended a concentration at which the product was to be used for washing farm dairy utensils. Tests were made on solutions of products at use-concentration and also at half this concentration.

Test organism. Escherichia coli type I (NCDO 555) was carried on nutrient agar slopes at 37°. For test purposes only the 4th to 8th daily subcultures from an original stock slope stored at 5° were used. Growth from a 24 hr slope was emulsified and suspended in 9 ml of quarter-strength Ringer's solution, shaken with glass beads and filtered through Whatman no. 4 paper. Then 2–3 ml were added to 120 ml of boiled glass-distilled water containing 20% (v/v) of commercial homogenized milk (Cousins, 1951). After mixing, the test suspension, which was prepared about 30 min before use, was divided into 2 equal parts.

Preparation of disinfectant solutions. Fresh solutions of the products were prepared for each day's tests in boiled glass-distilled water at double the concentrations finally required to allow for subsequent dilution in the test procedure. Similarly a hypochlorite-detergent solution as described for the Can Test was prepared. The disinfectant solutions, in 5 ml quantities, were distributed in duplicate in 1 oz McCartney bottles, and for controls sterile distilled water was used.

Test conditions. The disinfectants were tested in the presence of 10% of homogenized sterilized milk at 44° for a contact time of 2 min. The temperature and time corresponded to those in the Can Test.

Test procedure. One set of dilutions and a control bottle in randomized sequence, together with 60 ml of bacterial suspension, were placed in a 44° water bath with a stirrer to increase the rate of heat transfer. As soon as a control bottle similar to that containing the test bacterial suspension reached 44° (5–7 min) the test was started. To 5 ml of test solution, 5 ml of suspension were added; after exactly 2 min, 1 ml of the mixture was withdrawn and added to 9 ml of inhibitor (a solution containing 3% of Lubrol W and 2% of egg lecithin for QACs, or 0·1% of $Na_2S_2O_3.5H_2O$ for hypochlorite). After thorough shaking, the inhibited mixture was stored in iced water to await plating. The procedure was repeated with the remaining bottles of disinfectant, and with careful timing 11 solutions could be handled in 12 min. Using the duplicate bacterial suspension, the test procedure was repeated in a similar manner with the duplicate set of disinfectant dilutions. From preliminary experiments there was some evidence that keeping the bacterial suspension at 44° for more than 30 min reduced the resistance of the test organisms to disinfection. However, by using duplicate suspensions, each was at 44° for a maximum of 12 min.

From the inhibited mixture, 1 ml quantities of suitable decimal dilutions were plated in duplicate on nutrient agar and the colonies were counted after incubation at 37° for 48 hr. The randomized sequence was followed at each stage of the test.

RESULTS

The 14 materials were tested in 4 batches, each with a hypochlorite control, and usually with 4 replications of each dilution. The results (Table 5) for all the materials were reasonably consistent and no statistical analysis has been attempted. The products showed a wide range of efficiency, from a 'kill' of 99·99% at ½ use-concentration (Product 9) to almost complete lack of bactericidal effect, even at use-concentration (Products 2 and 14). Control counts for each experiment are shown in Table 6. The percentage survivors for hypochlorite (Table 6) show that dilution reduced the bactericidal efficiency more for this disinfectant than for QACs. Comparing Tables 5 and 6 it is seen that at ½ use-concentration 6 QACs (4, 7, 8, 9, 10 and 13) gave results better than hypochlorite and that 2 QACs (3 and 6) were as good as hypochlorite. Products 3 and 6, however, were not as good as hypochlorite at use-concentration. If the test were used routinely it would be desirable to include with each batch of products a QAC as reference standard to check that the resistance of the culture to QACs remained constant. For this work hypochlorite was used because it is the standard with which materials are compared in the Can Test.

Table 5. *Percentage survivors of* E. coli *after exposure for 2 min at 44° to QAC-detergent solutions in the presence of 10% milk*

QAC-detergent no.	Use-concentration			½ Use-concentration		
	Day 1	Day 2	Mean	Day 1	Day 2	Mean
1	0·87 0·65	1·06 0·63	0·80	41·1 47·4	44·6 55·9	47·3
2	>10 >10	81·7 91·5	86·6*	95·1 100	100 100	99
3	0·01 0·02	0·05 0·04	0·03	3·3 5·7	4·9 8·7	5·7
4	0·0001 0·001	0·0002 <0·0001	0·0003	0·11 0·08	0·28 0·15	0·16
5	0·47 0·25	0·40 0·44	0·39	45·2 41·0	42·2 46·8	44·6
6	0·02 0·002	0·04 0·02	0·02	9·4 11·5	7·6 7·3	8·5
7	0·0003 0·0001	0·0006 0·0003	0·0003	0·13 0·14	0·06 0·11	0·11
8	<0·0001 <0·0001	0·0001 <0·0001	<0·0001	0·02 0·03	0·02 0·01	0·02
9	<0·0001 <0·0001	No test ,,	<0·0001	0·02 0·01	No test ,,	0·01
10	<0·0001 0·0008	,, ,,	0·0004	0·03 0·06	,, ,,	0·05
11	0·004 0·004	,, ,,	0·004	34·3 24·2	,, ,,	29·3
12	0·24 0·18	0·08 0·22	0·18	40·8 44·2	44·3 30·3	39·9
13	0·0002 0·0002	0·0006 0·0001	0·0003	0·38 0·33	0·21 0·36	0·32
14	>10 >10	80·6 75·5	78·1*	100 100	100 100	100

* Excluding results for day 1.

Table 6. *Mean colony counts on controls, and percentage survivors of* E. coli *after exposure for 2 min at 44° to hypochlorite solutions in the presence of 10% milk*

Experiment no.	Mean colony count of control tubes (10/^{4}ml)	% survivors in hypochlorite solutions	
		Use-concentration (300 p/m av. Cl)	½ Use-concentration (150 p/m av. Cl)
1	367	<0·0001	5·5
2	435	<0·0001	8·5
3	332	0·0008	8·3
4	406	<0·0001	7·5
5	317	0·0002	5·5
6	353	0·0007	6·0
7	360	0·0003	5·3
	Means	0·0003	6·7

A Comparison of Suspension Test and Can Test Results

Eleven of the 14 materials tested by the Suspension Test were compared with hypochlorite plus detergent (H) in 3 series of Can Tests. The results for the remaining three products were extracted from two different series of tests. The derived mean colony counts/ml of rinse obtained with the Can Test for the QACs and H, and the corresponding percentage survivors obtained in the Suspension Test, are shown in Table 7. The QAC products are listed in order of effectiveness in each Can Test series. Products 3, 5, 6 and 7 were tested twice, and although results from different series are not strictly comparable the derived mean colony counts of these products are of the same order in the different series. In contrast to the behaviour of hypochlorite, the surface disinfecting efficiency of most of the QACs was reduced quite markedly by dilution.

Table 7. *Mean colony counts for cans treated with QAC-detergent solutions and hypochlorite plus Na_2CO_3, in the Can Test, compared with percentage survivors of* E. coli *in the Suspension Test*

Series	Product	Results for Can Test (derived mean colony count/ml of can rinse)		Results for Suspension Test (mean % survivors, *E. coli*)	
		Use-concn.	⅔ Use-concn.	Use-concn.	½ Use-concn.
1	8	6·33	63·9	<0·0001	0·02
	6	141	329	0·02	8·5
	7	136	1,240	0·0003	0·11
	1	240	10,300	0·8	47·3
	5	611	15,700	0·39	43·8
	Hypochlorite	14·2	19·2	0·0003	6·7
2	6	46	421	0·02	8·5
	7	57·2	3,370	0·0003	0·11
	3	241	3,110	0·03	5·6
	5	766	27,400	0·39	43·8
	11	3,350	80,900	0·004	29·3
	Hypochlorite	12·4	17·2	0·0003	6·7
3	9	0·15	1·62	<0·0001	0·01
	13	2·96	39·6	0·0003	0·32
	10	12·5	187	0·0004	0·05
	3	108	3,100	0·03	5·6
	2	60,400	175,000	86·6	100
	Hypochlorite	7·19	63·7	0·0003	6·7
	4	1,130	4,370	0·0003	0·16
	Hypochlorite	3	17	0·0003	6·7
	12	5,939	27,700	0·18	39·9
	14	35,100	38,900	78	100

While there is no well defined agreement between the results of the two tests, some relationship is apparent and this becomes more obvious when the materials are grouped according to their performance in the Suspension Test, and when Can Test results are expressed as 'pass' or 'failure' (Table 8). Of the group 2 materials (those giving more than 0·001% survivors at use-concentration and more than 1% survivors at ½ use-concentration) none passed the Can Test. However, products 4 and 7 in group 1, which showed good bactericidal properties in suspension, were poor surface disinfectants. One (4) was the only liquid QAC-detergent formulation, and the other (7) was the only product with a recommended use-concentration of less than 0·25%. It is probable that they were poor detergents. Details of the formulae of these commercial products, where known, are confidential, but it can be said that all those in group 1 had 200–300 p/m of QAC in the use-concentration and all except one contained alkyldimethylbenzylammonium chloride. In group 2 the QAC content of the test solutions varied from about 130–325 p/m.

Table 8. *The bactericidal efficiency of QAC-detergent solutions in the Suspension Test compared with their performance in the Can Test relative to hypochlorite plus* Na_2CO_3

	Product	Results for Suspension Test (mean % survivors of *E. coli*)		Results for Can Test (comparison* with hypochlorite)	
		Use-concn.	½ Use-concn.	Use-concn.	⅔ Use-concn.
Group 1	9	<0·0001	0·01	=	=
	8	<0·0001	0·02	=	=
	4	0·0003	0·16	X	X
	7	0·0003	0·11	=	X
	13	0·0003	0·32	=	=
	10	0·0004	0·05	=	=
Group 2	6	0·02	8·5	=	X
	3	0·03	5·6	=	X
	11	0·004	29·3	X	X
	12	0·18	39·9	X	X
	5	0·39	43·8	X	X
	1	0·80	47·3	X	X
	14	78·0	100	X	X
	2	86·6	100	X	X
Hypochlorite		0·0003	6·7		

* =, as good as hypochlorite; X, significantly less effective than hypochlorite.

Discussion

Comparison of the two tests, with a relatively small number of products, suggests that a suspension test could be of some value for screening QAC-detergents. The results in Table 8 serve as a guide to the level of bactericidal efficiency required of a material under the conditions specified in the Suspension Test if it is to compare favourably with the standard hypochlorite-detergent preparation in the Can Test. However, a product giving good 'kills' in the Suspension Test may not be a good surface disinfectant if it lacks detergent properties and is therefore unable to remove a dried milk film from the surface of the can. Unless bacteria are released and exposed few will be destroyed even by an effective disinfectant.

It is well known that many QACs are more effective in the alkaline pH range. Where the pH of a QAC solution is high (e.g. 10–11) by reason of the incorporation of a compatible alkaline detergent, the bactericidal efficiency of the QAC may well be enhanced and the solution may also have good detergent properties. Thus, for some QAC-detergent mixtures performance in suspension in the presence of 10% of milk could predict performance on a milk-soiled surface.

With other disinfectants, the Suspension Test may be much less reliable for initial screening. For example, reducing the pH of a hypochlorite preparation from 10·4 to 8 brings about a striking improvement in bactericidal properties in the Suspension Test but has little effect on surface disinfection; the performance of an iodophor in the Can Test was disappointing compared with its high activity in suspension (Cousins, Hoy & Clegg, 1959).

It is clear that detergent formulation may influence markedly the surface disinfecting properties of QACs, iodine and, to a lesser extent, chlorine. Therefore the Can Test may well prove useful for determining the relative parts played by detergents and disinfectants, in combination and separately, for cleansing milk-soiled surfaces.

It is possible that a suspension test may be more suitable for evaluating disinfectants intended for use on cleaned surfaces, but this can only be determined by comparing suspension test results with those obtained by a modified Can Test, in which the soiled cans are treated first with detergent and then with disinfectant. This modification has already been tried, with some success, but no comparative results are yet available.

The Can Test is now used to evaluate new detergent-disinfectant combinations intended for washing farm dairy utensils by hand. However, other methods of applying these materials may still require controlled field experiments, particularly where it is suggested that dismantling, brushing and the use of heat as a bactericidal process can be dispensed with except at infrequent intervals, for example circulation cleaning (Cuthbert, 1960) or immersion cleaning (Thiel, Clough & Clegg, 1955).

In such circumstances it might be necessary to determine the physical as well as the bacteriological condition of the equipment after repeated use and cleansing treatment; it would be difficult to devise laboratory tests simulating the condition of the components after perhaps 3 months of farm use.

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The present address of Professor L. F. L. Clegg is the Department of Dairy Science, University of Alberta, Edmonton, Alberta, Canada.

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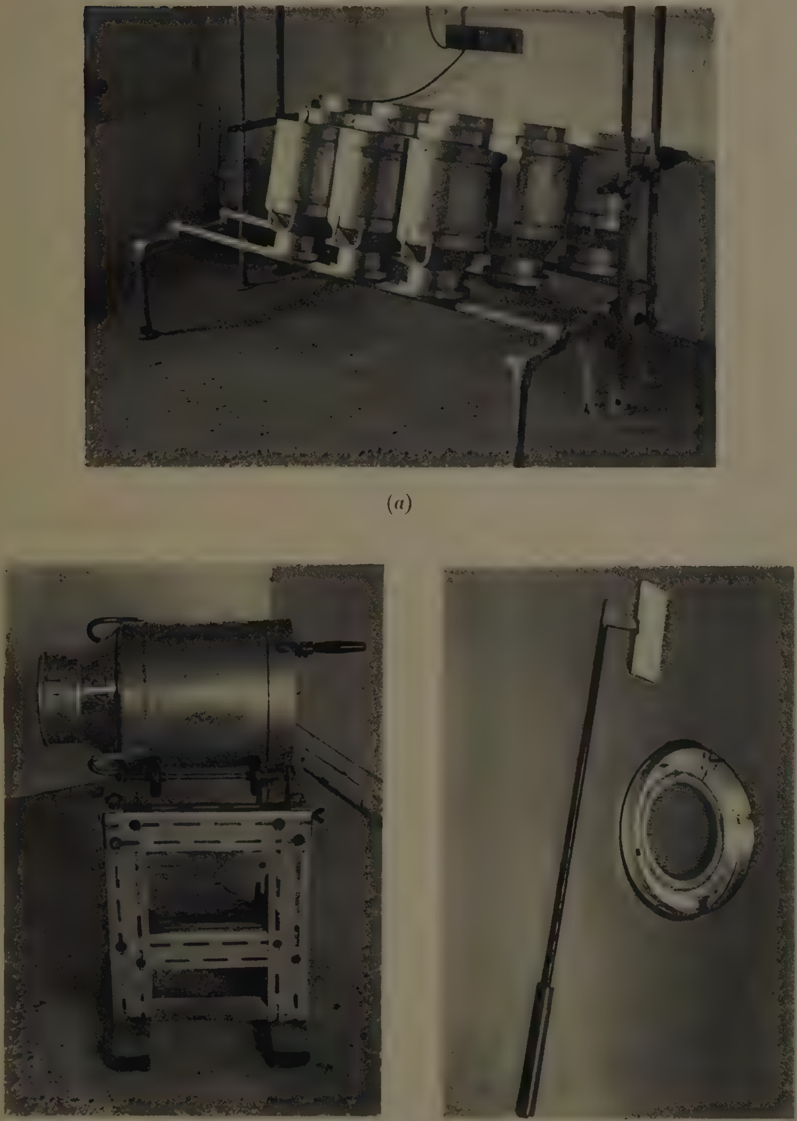

(*a*)

(*b*) (*c*)

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EXPLANATION OF PLATE

Equipment used in the Can Test.

(a) Rack with 12 soiled cans for overnight incubation.
(b) Can with handle attached and can roller.
(c) Perforated zinc lid and long handled squeegee.

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper I)

INTRODUCTORY REFLECTIONS ON THE *PSEUDOMONAS–ACHROMOBACTER* GROUP

BY M. INGRAM AND J. M. SHEWAN

Low Temperature Research Station, Cambridge and Torry Research Station, Aberdeen

WHEN EXAMINING the flora of natural habitats—air, soil or water, or the plant and animal materials in contact with them—one usually finds a high proportion of Gram-negative rods. Some of these, such as *Azotobacter*, are rather specialized organisms and are isolated only when appropriate special media are used; but we are here concerned with the generality of more ordinary types which grow on the usual media or even on rather poor media. They may form colonies of various colours, and can be nonmotile or have polar or peritrichous flagella. Those which form red or purple pigments are, in proportion, more common in air, soil and water, whereas on plant and animal materials kinds which produce the white, green or yellow pigments predominate: the latter are our particular concern here. Because of their nearly universal distribution, they are of great general importance. They are common on proteinaceous foods, and usually dominate the flora under cool conditions. Many members of the group are believed to play leading roles in water and soil, e.g. to '*Pseudomonas*' is allotted the function of returning protein nitrogen into circulation as ammonia under aerobic conditions. Moreover, many show great versatility in metabolism, so that no matter how refractory an organic material may appear, if it is broken down by bacterial action an organism of this group will almost certainly be involved. Many are plant pathogens and the 'green forms' include the well known human and animal pathogen *Pseudomonas aeruginosa*. Some of the others may also play a role in human and animal pathology.

Obviously, much research is being done with these bacteria on various topics, such as their natural occurrence in soil and aquatic environments, in foods, and in plant and animal diseases, and also on their biochemistry and taxonomy. These topics tend to lack sufficient connection, and the organizers of this Symposium thought it an opportune occasion to bring together workers in the field, to survey the progress made, to discuss some of the common problems, and perhaps to indicate lines for future work. All the topics mentioned are considered in later papers; and it seems certain that few groups of bacteria can be of such widespread interest.

The name *Pseudomonas* was first used for some of them about 1900 by Migula who, apart from general morphology, stressed the importance of flagellation as a means of differentiation. Somewhat later Orla-Jensen (1909) proposed another system of classification in which physiological characters were the prime means of differentiation, but it was severely criticized and did not come into general use. Nevertheless some of the physiological concepts were subsequently fairly successfully married with the morphological ones by Buchanan (1925) to form the basis of a classification in the first edition of the book familiarly known as Bergey's *Manual of Determinative Bacteriology.*

In the early editions of this invaluable (if much criticized) *Manual* those members of the Eubacteriales which were heterotrophic, had straight cells and did not form spores were called Bacteriaceae. The Gram-negative saprophytes of this family (excepting the cellulose digesters) were then divided according to colour: e.g. *Flavobacterium*, yellow; *Pseudomonas*, green, and those forming no pigment, *Achromobacter*. Division being on the basis of pigmentation, type of flagellation was not regarded as important, and each genus included peritrichous, polar and nonmotile species with, as time went on, some of undetermined flagellation also. As Table 1 shows, this arrangement was continued through the 2nd, 3rd and 4th editions of the *Manual*, the 4th edition being issued in 1934. Thus there was established a conception of the *Pseudomonas–Achromobacter* group as bacteria associated taxonomically as well as ecologically.

At that time there was in Britain little bacteriology other than medical; for example, in the eight-volume System of Bacteriology published by the Medical Research Council in 1931, *Achromobacter* does not appear in the index. As this situation has begun to change only comparatively recently, for readers of English the early history of the *Pseudomonas–Achromobacter* group has to be regarded mainly in the terms of Bergey's *Manual*. But in 1936 appeared the classification of Kluyver & van Niel, with a tentative phylogenetic basis. These authors criticized the Bergey system, in particular insisting on the importance of flagellation, suggesting that the peritrichous Bacteriaceae represented an evolutionary line different from that of the polar Pseudomonadaceae, both springing independently from cocci. They further suggested that *Pseudomonas* should be regarded as having a strictly oxidative metabolism.

These suggestions obviously induced radical changes of outlook. In the next (5th) edition of the *Manual* it was evidently no longer felt possible to include peritrichous and polar organisms in one genus. The peritrichous forms were removed from *Pseudomonas*, now a genus of polar species, the type being *Ps. aeruginosa*. Similarly, the species with polar flagella were removed from *Achromobacter*, which was now based on the peritrichous type species *A. liquefaciens* (see Table 1). In *Pseudomonas* the change was made by transferring five species, which in previous editions had been described as peritrichous, to the section 'flagella not reported' (some of them re-appeared in the 6th edition as 'polar'), and removing the polar species from *Achromobacter* to an appendix containing organisms of doubtful affinity regarded as probably belonging to the Pseudomonadaceae. The genus *Achromobacter*, now containing only peritrichous species, was transferred to the Bacteriaceae and that family was separated from the Pseudomonadaceae by the Coccaceae, thus implicitly following Kluyver & van Niel. Hence the genera *Pseudomonas* and *Achromobacter* were, in the 5th edition of the Manual, for the first time separated, and by hundreds of pages. The genus *Flavobacterium* was dealt with in a similar way and has remained alongside *Achromobacter* in subsequent editions.

Apart from these changes the general nature of the genera was not much altered in the 5th edition, as is evident from Table 1; but in the 6th further substantial changes were made. *Pseudomonas* was greatly enlarged: first, by the inclusion of nearly a hundred plant pathogens, whose status as distinct species is at present being seriously questioned, and second, by the admission for the first time of nonpigmented species, twenty-three in number. Of these twenty-three, no less than fourteen were from the nineteen polar species which had been excluded from *Achromobacter* in edition 5. The remaining nonpigmented species included a few which produced gas from sugars.

Table 1. *The distribution of species, in successive editions of Bergey's* Manual of Determinative Bacteriology, *between* Pseudomonas *and* Achromobacter

Genus and type of flagellation	Nos. of species in edition					
	2, 1926	3, 1930	4, 1934	5, 1939	6, 1948	7, 1957
Pseudomonas						
Polar	14	19	19	19	61	52
Peritrichous	4	5	5	0	0	0
Unknown	0	3	3	8	84*	92*
Nonmotile	2	4	4	4	3	5
Achromobacter						
Polar	9	17	19	0	0	0
Peritrichous	29	32	34	19	7	10
Unknown	0	13	13	9	0	0
Nonmotile	18	22	20	14	5	5

* Plant pathogens.

Alongside the green (or occasionally nonpigmented) plant pathogenic pseudomonads, Dowson's genus *Xanthomonas* was accepted for the yellow plant pathogens with polar flagella. One of the characters defining the genus was the production of acid from sugars, in a manner not specified, and it was included in the Pseudomonadaceae.

In the 6th edition, *Achromobacter* was further reduced by the removal of imperfectly described species to an Appendix. However, *Achromobacter* and *Flavobacterium* were joined in a new peritrichous family Achromobacteriaceae, together with *Alcaligenes*. The name *Alcaligenes* had long been used by medical bacteriologists for coli-aerogenes organisms which did not ferment lactose and therefore frequently turned litmus milk alkaline. Because the milk reaction is diagnostic for the genus, though it is in fact a character shown by other more or less related bacteria, *Alcaligenes* has received many non-lactose-fermenting species found in milk. In the 6th edition of Bergey's *Manual*, *Alcaligenes* was divided from *Achromobacter* on the basis that the species in the former never produced acid from hexoses, while those of the latter did—usually! Like *Achromobacter*, *Alcaligenes* included motile and nonmotile species.

In the decade between the 6th and the latest edition (the 7th, 1957) of the *Manual*, much has been learned about the biochemistry particularly of a few *Pseudomonas* strains. The findings are slowly being incorporated into schemes for differentiating groups or genera, and an example is the important though simple test of Hugh & Leifson (1953) which reveals whether acid is produced oxidatively or fermentatively (i.e. anaerobically) from sugars—a distinction which is not obvious in the customary fermentation test. The biochemical advances led to further subdivisions of the Pseudomonadaceae in the 7th edition of Bergey's *Manual*, and these again corresponded to suggestions made long before by Kluyver & van Niel: *Pseudomonas*, *Xanthomonas* and *Acetomonas* are defined as attacking carbohydrates oxidatively; *Aeromonas* and *Zymomonas* are reserved for the *Pseudomonas*-like organisms which attack sugars fermentatively with the formation of gas. The genera *Achromobacter*, *Alcaligenes* and *Flavobacterium*, which remain in the peritrichous Achromobacteriaceae in the 7th edition, are also characterized as attacking sugars oxidatively (if at all) and this distinguishes them from the fermentative Enterobacteriaceae.

While these ideas were being developed by the editors of Bergey's *Manual*, Prévot (1933) in France proposed a different scheme, the main features of which were the primary importance of morphology and the subordination of physiology to differentiation of species only. Thus, he insisted (Prévot, 1948) that it was wrong to group together, simply because they attack cellulose, anaerobes whose methods of sporulation are quite different.

Prévot's principles have however been curiously applied to the classification of the Pseudomonadaceae by Brisou (1958), who collaborated with him. This classification is notable in two respects, which are reminiscent of the early editions of Bergey's *Manual*. First, pigmentation, as a physiological character, is reinstated as the primary criterion. Second, an unusual attitude is adopted towards flagellation: the difference between polar and peritrichous flagellation is ignored; but, remarkably, it is then regarded (to quote Brisou's words) as ". . . impossible to include in the same genus flagellated species and species without locomotive apparatus". As a result, nonpigmented polar species of *Pseudomonas* rejoin the peritrichous species in *Achromobacter*; but nonmotile species (mostly from *Achromobacter*) are assembled in a new genus with the name *Acinetobacter*.

This last suggestion has something to commend it. The nonmotile strains most frequently isolated by ourselves and others are highly characteristic, very short or coccoid rods often occurring in pairs, in which form they might be taken for *Neisseria*—a name which one would not wish to apply to bacteria common on foods. These organisms correspond closely to those variously termed *Bacterium anitratum*, *Diplococcus mucosus*, *Mima*, or *Moraxella*. Though morphologically similar, the strains of this sort are biochemically diverse; and we do not yet know, for example, whether any general distinction can be made between nonmotile species of *Alcaligenes* and of *Achromobacter*, or between these and the types just mentioned. It is in this field, particularly, that more work is required at present.

It may be observed, even from this brief account, that the organisms which will occupy most of our attention today, *Pseudomonas* and *Achromobacter*, with their respectively related genera, are classified in Bergey's *Manual* in different orders—

the Pseudomonadales and Eubacteriales respectively—whereas in the Brisou–Prévot scheme they are in the same family; a radical difference which springs simply from the choice of a particular character as of primary importance, especially Brisou's emphasis on pigmentation rather than on the difference between polar and peritrichous flagellation. The relative emphasis to be attached to morphology and to physiology is of course a fundamental question which will certainly arise repeatedly in our discussions; but it seems difficult to decide the question at present even in a particular case. Thus, considering the decisions made by Brisou in adopting his system, while recent experience makes the difference between polar and peritrichous flagellation much less clear than has been thought, the resemblance between green and white pseudomonads now appears so close (as later papers in this Symposium will show) as to reduce the difference in pigmentation to a plainly low order of significance.

What is clear is that there is still much to gain from the study of metabolism and, in the most recent edition of Bergey's *Manual*, it is suggested that differentiation of the genera may depend primarily on biochemical characters. Thus both *Pseudomonas* and *Acetomonas*, when attacking glucose, produce 2-ketogluconic acid; but the latter can oxidize ethanol and tolerate high acidity. Because various oxidative pathways have already been revealed in a few members of this group of organisms, further differentiation may be possible, once appropriate tests sufficiently simple for routine use have been devised. As will be seen later, like Buttiaux & Gagnon (1959) we have lately begun to use an oxidase test as an aid; and a further useful and significant test for ammonia production from arginine has just been devised (Thornley, 1960).

Of the remaining polar-flagellate, Gram-negative, nonsporing rods, two other groups call for further brief mention: *Xanthomonas* and *Vibrio*. The majority of *Xanthomonas* are oxidative and distinguished from *Pseudomonas* mainly by their pigmentation and by pathogenicity for plants. There are however two yellow *Xanthomonas* species, named in the current *Manual*, which produce gas from sugars and hence resemble *Aeromonas*, while some of the pathogenic species are almost white, resembling *Pseudomonas*, and there are yellow strains which were isolated as saprophytes. It appears that, like the plant pathogenic pseudomonads, *Xanthomonas* probably contains no more than the pathogenic members of a larger and more heterogeneous group which requires wider study, and may differ mainly in colour from *Pseudomonas* and *Aeromonas*.

The *Vibrio* group, though it contains nonpigmented rods with polar flagella, has been removed from the Pseudomonadaceae in Bergey's *Manual* and placed in the family Spirillaceae. The genus *Vibrio* was created around well-known animal pathogens with curved cells; but this curvature, which for saprophytic strains is almost the only diagnostic feature, is by itself an unsatisfactory character and the genus as at present understood almost certainly contains a heterogeneous collection of species, some of which ought probably to be near *Pseudomonas* (see Shewan, Hobbs & Hodgkiss, 1960).

In all this, little has been said about the separation of species, because the more experienced workers in this field have for the present ceased trying to identify them.

It seems certain that most of the *Pseudomonas* and *Achromobacter* species described in the current lists of Bergey or of Brisou are illusory; in this connection one may recall the recent monograph by Rhodes (1959), in which it is suggested that the species of *Pseudomonas* (exclusive of plant pathogens) should be reduced to three, not very readily separable. With *Achromobacter* species the situation is even worse, for these have been little investigated and the type species cannot be found.

Overshadowing all this are the difficulties in defining even genera, which spring from misgivings about the validity of characters like flagellation and pigmentation, long thought to be stable and fundamental. It is a situation ripe for agnostic application of the Adansonian techniques of Sneath (1957); and it appears that, in this group of bacteria especially, it may be necessary to relinquish the old conception of the genus as a distinct systematic unit characterized by basic homogeneity in morphology and metabolism.

As far as one of us (J.M.S.) is concerned, this paper was prepared as part of the programme of the Torry Research Station of the Department of Scientific and Industrial Research.

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper II)

A DETERMINATIVE SCHEME FOR THE IDENTIFICATION OF CERTAIN GENERA OF GRAM-NEGATIVE BACTERIA, WITH SPECIAL REFERENCE TO THE PSEUDOMONADACEAE

BY J. M. SHEWAN, G. HOBBS AND W. HODGKISS
Torry Research Station, Aberdeen

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1. INTRODUCTION

THE IDENTIFICATION and classification of bacteria isolated from marine environments, including fresh and spoiling fish, have been subjects of study in our laboratory for many years and we have found that the majority of our isolates are Gram-negative asporogenous rods. Wherever these bacteria are encountered, as in studies concerned with aquatic environments generally, soils, foods, and plant and animal diseases, investigators are generally agreed that classification presents considerable difficulty. Widely divergent views on taxonomy are reflected in the various proposed systems of classification (Ingram & Shewan, 1960), and resolution of the difficulty has been delayed by the lack of readily applicable discriminatory tests. However, over the past decade the work of identification has been greatly eased by the development of new tests as well as by improvements in existing apparatus, e.g. phase contrast and electron microscopes.

Precise identification of organisms is of course not simply of academic interest; it often has great practical significance. In our own field, for example, it is important to know whether the *Pseudomonas* spp. causing fillet spoilage are present on the fish in the sea, in the ice used to chill the fish, or are picked up from the filleting benches. Studies of the changes in the bacterial flora of fish during storage for up to about 16 days in ice involve the handling of several hundreds of micro-organisms, and this led us to seek speedier methods of identifying large numbers of isolates. In the first instance differentiation at the generic level was the aim. Our early results (Shewan *et al.* 1954) suggested some broad differentiations of groups corresponding roughly to the genera *Pseudomonas*, *Achromobacter* and *Vibrio* and the family Bacteriaceae of Bergey's *Manual of Determinative Bacteriology*, 7th ed. (Breed, Murray & Smith, 1957), and from the experience gained since then the scheme presented here and now in use in our laboratory was developed.

2. The Determinative Scheme

The medium used for isolation and cultivation of the bacteria investigated was 'Oxoid' Lab-Lemco nutrient agar made up either with tap water or with 75% of aged sea water as required (ZoBell, 1946). The original isolate was plated to obtain a pure culture and then put through the tests outlined in Table 1. The colony appearance, the Gram reaction, the morphology and the behaviour in the oxidase test (Kovacs, 1956) were obtained from the agar plate culture, and observations of motility and morphology (under phase contrast) were done on a Lab-Lemco broth culture. This latter culture was also used to surface seed an agar plate for the sensitivity tests described by Shewan *et al.* (1954) and to inoculate two tubes of the medium of Hugh & Leifson (1953), one of which was incubated anaerobically under paraffin. The production of diffusible fluorescent pigment was determined using either the medium of King *et al.* (1954) or that of Paton (1959). When growth occurred in the latter medium, the production of 2-ketogluconic acid was recorded using an aniline oxalate or aniline phthalate reagent. A sheet of chromatography paper (Whatman no. 1) was impregnated with a saturated solution of recrystallized aniline oxalate or phthalate (Hough *et al.* 1950) and the culture heavily spotted on the paper, which was then heated in an oven at 105° for 2–3 min. A red spot indicated the presence of 2-ketogluconic acid. Occasionally the gluconate (as also glucose) appeared to be dissimilated beyond the 2-ketogluconic acid stage and this gave rise to yellow colours with the aniline reagent (Floodgate, pers. comm.). For the detection of intracellular pigments (e.g. those of flavobacteria) a nutrient agar

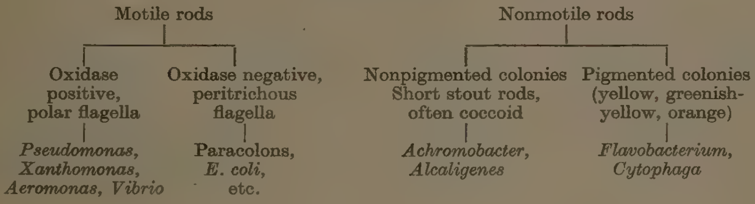
Table 1. *A summary of the tests used in the determinative scheme*

Medium	Observation
Nutrient agar	Colony appearance Gram stain Morphology Oxidase test (Kovacs, 1956) Antibiotic and O/129 sensitivity tests (Shewan *et al.* 1954)
Nutrient broth	Motility, morphology
Medium of King *et al.* (1954) Medium of Paton (1959)	Diffusible fluorescent pigments detected under U.V. light
Medium of Paton (1959)	2-ketogluconic acid formation
Medium of Hugh & Leifson (1953)	Dissimilation of carbohydrates
Nutrient agar + 30% skim milk	Pigment production by *Flavobacterium* spp.
Nutrient agar slope	Flagella stain (Casares-Gil)

incorporating 30% of skim milk is used. In motile cultures the type of flagellation was determined by the use of a modified Casares-Gil flagella stain (Manual, 1957) or by electron microscopy. Most of our isolates were psychrophiles and the tests were normally carried out at 20°; but they are equally applicable to mesophiles.

On the basis of the above tests the broad groupings outlined in Table 2 were obtained. It is important to stress, however, that the scheme was devised primarily for the organisms from the special environment concerned, mainly fresh and spoiling marine fish, although we have tested the scheme with many strains isolated from different environments by other workers. Thus the only members of the Enterobacteriaceae we normally encountered were 'paracolons' and *Escherichia coli*. We have thus had no experience with, for instance, the *Shigella* group, and it is possible that they would give rise to difficulties in our scheme. But in our experience the oxidase test of Kovacs (1956) is invaluable for the differentiation of the Enterobacteriaceae which we encounter from members of the Pseudomonadaceae, and this seems to be true for the Enterobacteriaceae generally, according to Ewing & Johnson (1960). The Enterobacteriaceae are of course being deliberately excluded from our present discussion, as are other Gram-negative rods such as the members of the genera *Azotobacter*, *Acetobacter* and *Cellulomonas*, which are not normally isolated except by the use of special media.

Table 2. *A broad grouping of organisms on the results of the tests in Table* 1


Motile rods
Oxidase positive, polar flagella
Pseudomonas, *Xanthomonas*, *Aeromonas*, *Vibrio*
Oxidase negative, peritrichous flagella
Paracolons, *E. coli*, etc.
Nonmotile rods
Nonpigmented colonies Short stout rods, often coccoid
Achromobacter, *Alcaligenes*
Pigmented colonies (yellow, greenish-yellow, orange)
Flavobacterium, *Cytophaga*

3. Discussion of the Groups

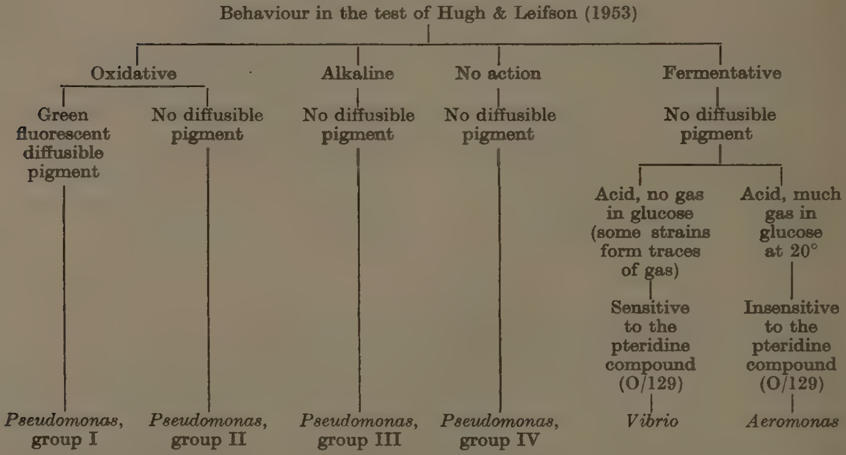
(a) *The polar-flagellate, oxidase positive group*

This comprises members of the genera *Pseudomonas*, *Aeromonas* and *Vibrio*. Some members of the genus *Xanthomonas* also fit in here, but at present our experience is too limited for any general conclusions to be drawn.

By means of the medium of Hugh & Leifson (1953) these organisms can be differentiated into those which give (1) an oxidative reaction (*Pseudomonas* spp.); (2) no reaction (*Pseudomonas* spp.); (3) an alkaline reaction (*Pseudomonas* spp.) and (4) a fermentative reaction (*Aeromonas* and *Vibrio* spp.) (Table 3).

(i) Pseudomonas *spp.* Since the oxidative *Pseudomonas* spp. can be further subdivided on the basis of the presence or absence of fluorescence under ultraviolet light, we therefore postulate four groups of *Pseudomonas* spp. Group I includes those, such as *Pseudomonas fluorescens* and *Ps. aeruginosa*, which are oxidative and produce a diffusible fluorescent pigment. Group II strains attack glucose oxidatively, but in our hands never produce a diffusible fluorescent pigment. Group III strains produce no diffusible pigment and have no action on glucose, but produce an alkaline reaction in Hugh & Leifson's medium, and group IV strains produce no change at all.

Table 3. *A grouping of the Gram-negative asporogenous rods, polar-flagellate, oxidase positive and not sensitive to 2·5 i.u. of penicillin, on the results of four other tests*


Behaviour in the test of Hugh & Leifson (1953)
Oxidative
Alkaline
No action
Fermentative
Green fluorescent diffusible pigment
No diffusible pigment
No diffusible pigment
No diffusible pigment
No diffusible pigment
Acid, no gas in glucose (some strains form traces of gas)
Acid, much gas in glucose at 20°
Sensitive to the pteridine compound (O/129)
Insensitive to the pteridine compound (O/129)
Pseudomonas, group I
Pseudomonas, group II
Pseudomonas, group III
Pseudomonas, group IV
Vibrio
Aeromonas

Further subdivision of groups I and II may be possible, since both contain strains which appear to effect the further breakdown of 2-ketogluconic acid.

Recently Sneath (1957*a*) has suggested that the most logical way of classifying bacteria is to compare the overall similarities of the organisms, applying the Adansonian principle of giving equal weight to each character. Stewart (1958), at the Torry Research Station, has recently studied in detail about 60 organisms isolated from marine sources by various workers, and on the basis of the determinative scheme outlined above classified them as *Pseudomonas* spp. The overall similarities of about 80 tests for 55 of these organisms have been calculated by G. D. Floodgate of the Torry Research Station, using the formula of Sneath (1957*b*). The results (Fig. 1) show that although there is some overlapping three groups of *Pseudomonas* can be differentiated, and these correspond generally to the organisms of groups I, II, and III and IV together. Of the recently described strains of *Pseudomonas* available to us, *Pseudomonas aureofaciens* (Kluyver, 1956) fits into group III, *Ps. fragi* (from the American Type Culture Collection) into group II and *Ps. rubescens* (Pivnick, 1955) into group IV.

(ii) Aeromonas *and* Vibrio *spp*. These are differentiated (Table 3) from the *Pseudomonas* species by their fermentative action upon glucose. *Aeromonas* spp. are characterized by the production of both acid and gas from glucose (at 20°) whereas *Vibrio* spp. ferment glucose anaerogenically. Moreover, only the latter are sensitive to 2:4-diamino-6:7-di-*iso*propylpteridine (O/129) (Shewan *et al.* 1954).

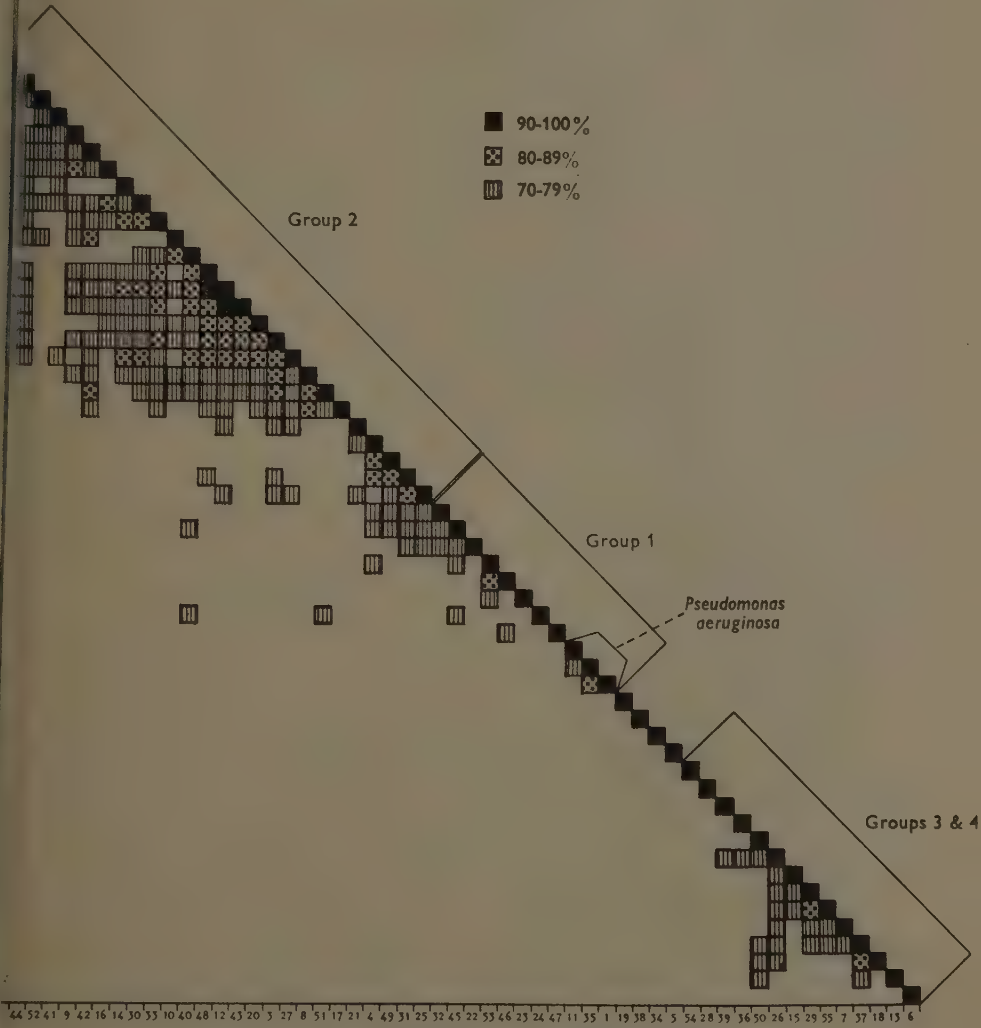


Fig. 1. A diagrammatic representation of the similarity values (% S values of Sneath, 1954) among 55 strains of *Pseudomonas*, obtained by the use of 80 features.

Stevenson (1959) has questioned the validity of the genus *Aeromonas* and has suggested that all its members could be regarded as nonchromogenic species of the genus *Serratia.* Several authors, Stevenson points out, have stated that flagellation in the genera *Aeromonas* and *Serratia* may vary according to the conditions of culture, and he believed that his *Aeromonas margarita* was in reality a nonchromogenic strain of *S. marcescens.* '*Aeromonas margarita*', however, is peritrichous (Plate 1, a) and this along with its biochemical characteristics indicates that it is a typical member of the Enterobacteriaceae. Members of the genus *Kluyvera*, defined as consisting of polar-flagellate Gram-negative rods which attack glucose fermentatively (Asai *et al.* 1956), and the so-called polar-flagellate member of the Enterobacteriaceae (Cook, 1958) (Plate 1, b), were also found to be peritrichous.

Two well recognized organisms, *Pseudomonas formicans* (Crawford, 1954) and *Bacterium salmonicida*, both regarded by many workers (Griffin, 1953; Snieszko, 1953) as *Aeromonas* spp., appear to occupy anomalous positions in our suggested scheme. *Ps. formicans* is a Gram-negative, oxidase positive, polar-flagellate asporogenous rod, which is fermentative in Hugh & Leifson's medium, forms no gas from glucose and yet is insensitive to the pteridine compound (O/129). In many supposedly key characters, e.g. formation of 2:3-butanediol and production of gas from glucose, it would appear to be excluded from the genus *Aeromonas*, and from its morphology and its insensitivity to the pteridine compound from *Vibrio* also. *Bacterium salmonicida* is also a Gram-negative rod, oxidase positive, forming acid and gas from glucose and fermentative in Hugh & Leifson's medium, but is nonmotile. Moreover, unlike *Aeromonas* spp. it is Voges-Proskauer negative.

Hugh & Leifson (1953) suggested that only fermentative organisms be included in the genus *Vibrio*. *V. percolans* and *V. cuneatus*, they pointed out, were not related either morphologically or physiologically to *V. comma* (Leifson, 1960). We also would exclude *V. cyclosites*, *V. neocistes* (Hugo & Rogers, 1958), *V. jamaicensis* (Caselitz, 1955), and many others listed as vibrios in some Culture Collections, on morphological and physiological grounds. In our experience most of these organisms are *Pseudomonas* spp. of the nonpigmented groups. *V. jamaicensis*, as Caselitz (1958) later pointed out, is an *Aeromonas* sp.

A new genus, *Lophomonas*, has been suggested for some organisms formerly known as *V. alcaligenes* (Galarneault & Leifson, 1956); but from the data presented these fit into our suggested group IV of *Pseudomonas*. On the other hand, certain organisms formerly described as *Pseudomonas* spp. should on morphological and physiological grounds be placed in the genus *Vibrio*, for example *Ps. ichthyodermis* (Hodgkiss & Shewan, 1950) and *Achromobacter ichthyodermis* (Wells & ZoBell, 1934; ZoBell & Wells, 1934).

V. comma, fresh water vibrios of the paracholera group, *V. foetus*, some organisms pathogenic for fish (Rucker, 1959; Smith, 1961) and *V. anguillarum* (Bagge & Bagge, 1956), all fit into the *Vibrio* group of our determinative scheme.

Previously it had always been difficult to differentiate the saprophytic vibrios and the vibrios causing disease in poikilothermic animals from other Gram-negative, polar-flagellate rods. This was usually done on the basis of the curvature of the cell, a quite unsatisfactory criterion. The fermentative character of their attack on carbohydrates, together with their sensitivity to the pteridine compound O/129, are thus valuable additional criteria. It could be added that in addition to the curvature of the cell, *V. comma* was characterized by the pleomorphism of the cells and in particular by the presence of round or coccoid bodies (Hammerl, 1906; Henrici, 1925). Quite recently Hallock (1959) has claimed that this is a distinguishing feature of the *Vibrio* group as a whole and more attention might be given to this character than hitherto. Coccoid bodies have been described in certain luminous bacteria (Johnson & Gray, 1949), which on our criteria have been classified as *Vibrio* spp. (Spencer, 1955), and they are a regular feature of the 'gut-group' vibrios of Liston (1955) (Plate 1, c and d).

(iii) Spirillum *spp.* Members of the genus *Spirillum* have been infrequently encountered in our field. It is considered that morphology alone, including the bipolar tufts of flagella (Williams, 1960), is sufficiently characteristic for identification. Other important features of members of this genus are sensitivity to penicillin, but not to the pteridine compound O/129, and no action upon glucose in Hugh & Leifson's medium.

In the 6th edition of Bergey's *Manual* (Breed, Murray & Hitchens, 1948) the *Vibrio* spp. were included in the Tribe Spirillae of the Family Pseudomonadaceae, whereas in the 7th edition (Breed, Murray & Smith, 1957) they form part of the separate Family Spirillaceae. In our determinative scheme, however, the *Vibrio* spp. appear to be more closely allied to the *Pseudomonas* spp. than to the *Spirillum* spp.

(b) *The nonmotile nonpigmented group*

In Table 2 the *Achromobacter* spp. are recorded as being nonmotile and nonpigmented, despite the fact that the type species, *Achromobacter liquefaciens*, is defined in the 7th edition of Bergey's *Manual* (Breed, Murray & Smith, 1957) as being peritrichous. However, owing to the absence of the original culture from any known collection and to the paucity of data concerning it in Bergey's *Manual*, it cannot be identified with any degree of certainty. Moreover, over the past ten years, in which many thousands of isolates have been screened at the Torry Research Station, we have never once encountered an organism which is peritrichous and yet possesses the typical morphology given in the original description (Eisenberg, 1891 and Frankland & Frankland, 1894). Breed was well aware of the difficulty arising from the absence of the type strain and in a private communication in 1947 stated that he believed an organism, corresponding to the original strain, had at last been isolated. However, no description of such a culture has yet appeared in the literature. The organism recently described as *Achr. liquefaciens* (Youatt, 1954; Skerman *et al.* 1958) has not been available to us, nor have the mesophilic *Achromobacter* spp., including one designated *Achr. liquefaciens*, of Rush (1947). Buttiaux & Gagnon (1959) have also recently concluded that members of the genus *Achromobacter* are nonmotile, since they never once encountered a peritrichous organism corresponding to *Achr. liquefaciens*, despite the fact that they were working with organisms isolated from very widely differing environments. A motile organism with peritrichous flagella, isolated by Kimata & Kawai (1953) and originally named *Achr. histamineum*, is fermentative in its action on glucose, producing acid and gas, and has recently been assigned by Kimata *et al.* (1958) to the genus *Proteus*.

We would define the *Achromobacter* group as being composed of nonmotile, nonpigmented, short, stout or coccoid rods, occurring singly, in pairs or short chains, forming grey or off-white slightly opaque colonies on agar, sensitive to penicillin, and usually biochemically inactive. In Hugh & Leifson's medium most strains produce an alkaline reaction, a few no reaction, and some strains are oxidative. In general these organisms appear to correspond to the genus *Acinetobacter* proposed by Brisou & Prévot (1954).

Achromobacter spp. are distinguished from *Pseudomonas* spp. primarily on motility and morphology, and they are usually less active biochemically. In addition most strains are sensitive to penicillin, whereas the majority of *Pseudomonas* strains are not.

(c) *The nonmotile pigmented group*

Although the genera *Flavobacterium* and *Cytophaga* are not being considered in this Symposium, for the sake of completeness they merit some mention in relation to the determinative scheme outlined in Table 2.

All the flavobacteria and many of the cytophagas are characterized by the formation on agar of yellow to orange translucent colonies. Pigmentation is most easily detected on a nutrient agar containing 30% of skim milk. This not only appears to enhance pigmentation but makes its detection easier.

Morphologically the cells of *Flavobacterium* spp. are generally longer and more slender than those of *Achromobacter* spp. They occasionally form filaments in fluid media, and the cells are usually less refractile under phase contrast. Although in Table 2 *Flavobacterium* spp. are designated nonmotile, a few motile strains have been encountered.

Cytophaga spp. generally are defined in Bergey's *Manual*, 7th ed. (Breed, Murray & Smith, 1957) as thin rods, often with pointed ends, which show flexing and gliding motility; hence on agar plates the colonies have a characteristic diffuse margin, spreading outwards and into the agar. Recent experience at the Torry Research Station (Hayes, 1960) has shown that the differentiation of *Flavobacterium* spp. from the yellow to orange *Cytophaga* spp. is by no means as clear cut as the definitions in Bergey's *Manual* imply, and more work will have to be done before the position can be clarified.

4. THE EFFECTIVENESS OF THE SCHEME

The determinative scheme outlined above and set out in detail with regard to the major groups in Tables 4 and 5, follows closely the system of Bergey's *Manual* (Breed, Murray & Smith, 1957) in that the genera all have the definitive characters given there. It has now been in use at the Torry Research Station for a number of years, although naturally refinements and adjustments are constantly taking place. During this time several workers have examined thousands of isolates, mainly from marine sources, and the classification of most of these cultures at the generic level has been accomplished. In addition we have examined more than 200 named strains from Type Culture Collections and individual workers; these have included *Pseudomonas*, *Achromobacter*, *Vibrio*, *Aeromonas*, *Xanthomonas* and *Flavobacterium* spp. In almost every instance we have been able to identify them unequivocally by means of our scheme, although in some cases this classification has differed from that originally given. In addition our scheme has been in use for some time in other laboratories, and other workers to whom the scheme has been demonstrated have published data (e.g. Browne & Weidemann, 1958) which show its general usefulness.

Table 4. *An outline of the determinative tests applied to certain Gram-negative asporogenous rod-shaped organisms*

Motility	Morphology	Colony appearance*	Penicillin†	Behaviour in Hugh & Leifson's medium‡	Kovacs' oxidase test‡	Growth at 37°	Genus, species or type
—	Short stout rods 0·8 × 1·0–1·5 μ and coccal forms	Grey to greyish-white, slightly opaque	++ or +	Alkaline or oxidative	N.A.	—	*Achromobacter* *Alcaligenes*
—	Slender rods, occasionally filamentous	Yellow to orange	—	N.A.	N.A.	—	*Flavobacterium*
+	Slender rods	Yellow-orange	—	N.A.	N.A.	—	*Flavobacterium*
+	Slender straight or curved rods	Translucent, colourless, diffusible fluorescent pigment ±	—	Oxidative	+	— or +	*Pseudomonas*
+	Slender, straight or curved rods	Translucent, colourless, occasionally pigmented	—	Alkaline or no action	+	—	*Pseudomonas*
+	Slender rods, straight or curved	Translucent, colourless	—	Fermentative, gas abundant	+	+	*Aeromonas*
+	Slender, straight or curved rods	Translucent, colourless, occasionally yellow pigmented	—	Fermentative with no gas or (very seldom) traces	+	—	*Vibrio* (marine origin)
+	Straight rods	Translucent, colourless	—	Fermentative, gas abundant	—	+	Paracolon *E. coli*

* ±, may or may not be present. † Sensitivity to 2·5 i.u.; ++, very sensitive; +, sensitive; —, insensitive; ‡ N.A., test not applied.

We need hardly stress that the scheme as it now stands is a determinative one and does not claim to express any phylogenetic relationships between groups. We are well aware of its present deficiencies and only future work will show whether these can be removed without altering its general pattern.

Table 5. *An outline of the behaviour of certain members of the family Pseudomonadaceae, and of the genus* Spirillum, *in certain determinative tests*

Genus or group	Behaviour in Hugh & Leifson's carbohydrate medium	Sensitivity* to		
		Penicillin	Terramycin	Comp. O/129
Pseudomonas group I, green fluorescent pigment formed	Oxidative, acid only	–	–	–
Pseudomonas group II,† no fluorescent pigment	Oxidative, acid only	–	+	–
Pseudomonas group III,‡ no fluorescent pigment	Alkali formed aerobically	–	+	–
Pseudomonas group IV, no fluorescent pigment	No action aerobically or anaerobically	–	+	–
Aeromonas (Kluyver & van Niel)	Fermentative, acid with abundant gas	–	+	–
Vibrio	Fermentative, but acid only	–	+	+
'Gut-group' vibrios (Liston) and related luminous types (Spencer)	Fermentative, acid with little gas	–	+	+
Spirillum	No action	+	+	–

* +, sensitive; –, insensitive. Penicillin, 2·5 i.u.; Terramycin, 10 mg.

† *Xanthomonas* spp. give the same reactions. ‡ See text.

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EXPLANATION OF PLATE

(a) *Serratia margarita* (Stevenson, 1959), culture SL 10a. Electron micrograph, Au-Pd shadowed, of cells from a 24 hr agar slope culture at 20°, ×13,300.

(b) Culture NCTC 9960 (Cook, 1958). Cells from a 24 hr agar slope culture at 20°, modified Casares-Gil flagella stain, ×1,330.

(c) 'Gut-group' vibrios (Liston, 1955), showing curved rods and the characteristic round bodies. Phase contrast, ×800.

(d) 'Gut-group' vibrios (Liston, 1955), showing rounded bodies in culture from sea water agar. Phase contrast, ×800.

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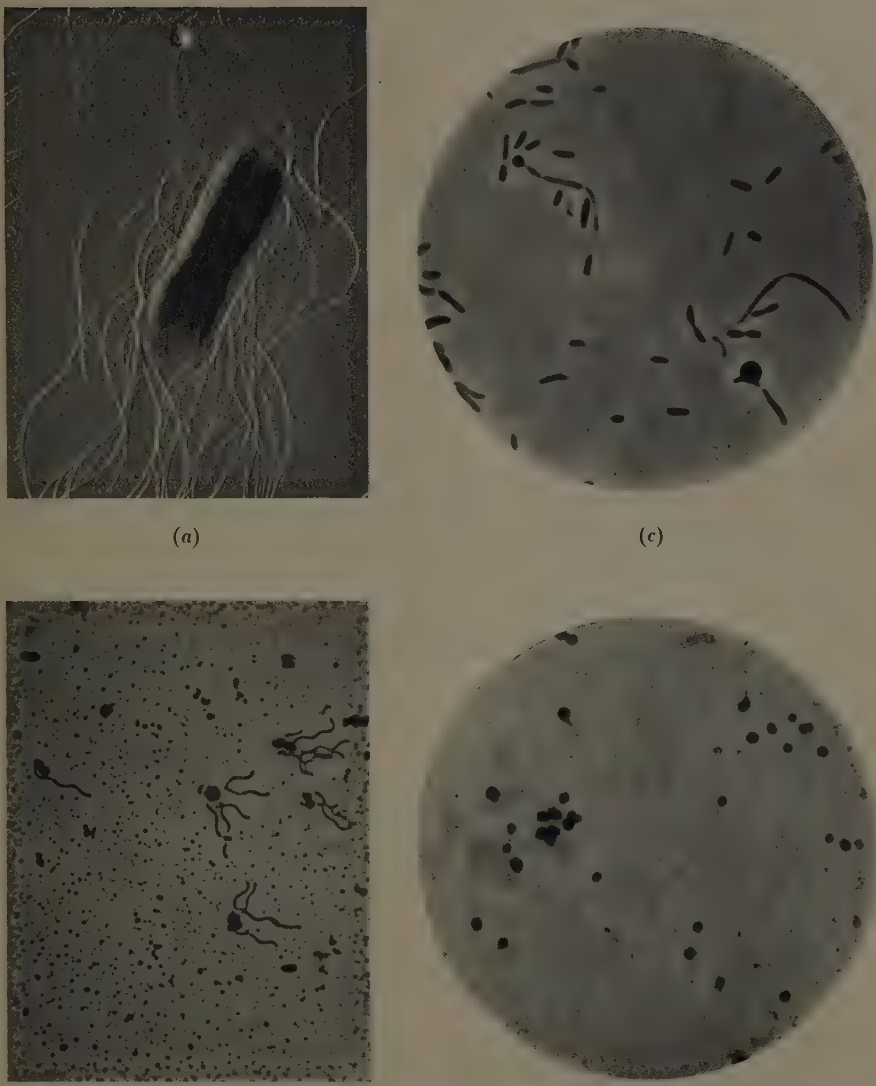

(*a*) (*c*)

(*b*) (*d*)

Shewan, J. M., Hobbs, G. & Hodgkiss, W. — A Determinative Scheme for the Identification of Certain Genera of Gram-Negative Bacteria, with Special Reference to the Pseudomonadaceae. Plate 1

Liston, J. (1960). *J. appl. Bact.* **23** (3), 391–394.

(Symposium on *Pseudomonas* and *Achromobacter*: Contribution relating to Paper II)

SOME RESULTS OF A COMPUTER ANALYSIS OF STRAINS OF *PSEUDOMONAS* AND *ACHROMOBACTER*, AND OTHER ORGANISMS

By J. LISTON
University of Washington, College of Fisheries, Seattle 5,
Washington, U.S.A.

I would like to introduce some of the results of an investigation into the taxonomy of organisms of the *Pseudomonas-Achromobacter* group using the Sneath computer method (Sneath, 1957), which has been under way for some time at the College of Fisheries, University of Washington, Seattle, U.S.A. Most of the work which has been done by Dr. Colwell and myself has been concerned with pseudomonads from marine sources, but we have also included in our analyses named and unnamed bacteria from other sources.

The results of our analysis of a collection of organisms, most of which were obtained from type culture collections (but completely retested in our laboratory) are shown in Fig. 1. Clearly the Sneath method has worked very well in separating groups of organisms which are generally recognized to be taxonomically dissimilar, e.g. *Bacillus*, the *coli-aerogenes* group and *Pseudomonas*.

In relation to the theme of the present meeting, the following two points, arising from the diagram, are probably most important. First, most of the so-called *Vibrio* spp. tested fell into the *Pseudomonas* group. This is in accord with our findings and those reported earlier by Hugh & Leifson (1953), that such organisms as *V. percolans* and *V. cuneatus* have all the characteristics of an oxidative *Pseudomonas* and none that could define them as *Vibrio*. *V. metschnikovii*, which alone fell outside the *Pseudomonas* group in our analysis, has some fermentative abilities but little cell curvature and forms a small subgroup with the anaerogenic species *Aeromonas formicans* at a rather low level of similarity. This was the nearest thing to a *Vibrio* group which our analysis revealed.

Second, the aerogenic *Aeromonas* grouped with the Enterobacteriaceae rather than with the Pseudomonadaceae. An analysis of intergroup relationships indicates that a natural taxonomic link may exist between the *Pseudomonas* and the *coli-aerogenes* groups by virtue of the *V. metschnikovii–A. formicans* group (nearest *Pseudomonas*) and the aerogenic *Aeromonas* group (nearest *coli-aerogenes*).

Fig. 2 shows the preliminary results of an analysis of 80 pseudomonad or related strains. Using tentatively a 60% S value for genus level (which seems from previous work to be reasonable), the *Pseudomonas* group proper appears to end at *Ps.*

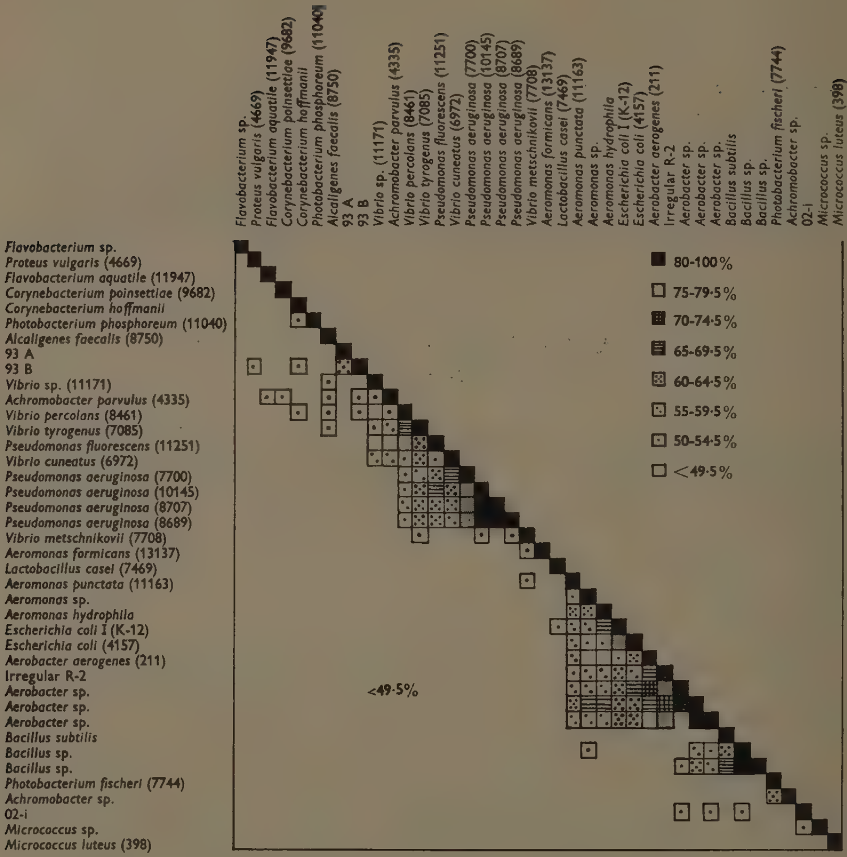

Fig. 1. A diagrammatic representation of the similarities between the members of a collection of organisms mostly obtained from culture collections.

pavonacea. Green pigmented organisms seem to cluster together as do also, but to a lesser extent, sea water requiring organisms. The largest single group, that of organisms related to *Ps. aeruginosa* (from PS 241 to *V. percolans*), in general form pigment, are biochemically active and do not require sea water. Possibly two other groups are indicated by these data, one comprising predominantly nonpigmented organisms, rather inactive biochemically and not requiring sea water (E 91a–1 to E 74b) and the other psychrophilic organisms, all somewhat inactive biochemically and requiring sea water (JL 224 to PS 247), of which some members may produce a soluble brown pigment. It is interesting to speculate whether these three groups correspond with the groups just delineated by Shewan (see Shewan, Hobbs &

Hodgkiss, 1960). However, it should be noted that the results reported here are preliminary: the intra- and intergroup analysis had not been completed when this diagram was made and, when it has, changes in the number and identity of the groups may appear.

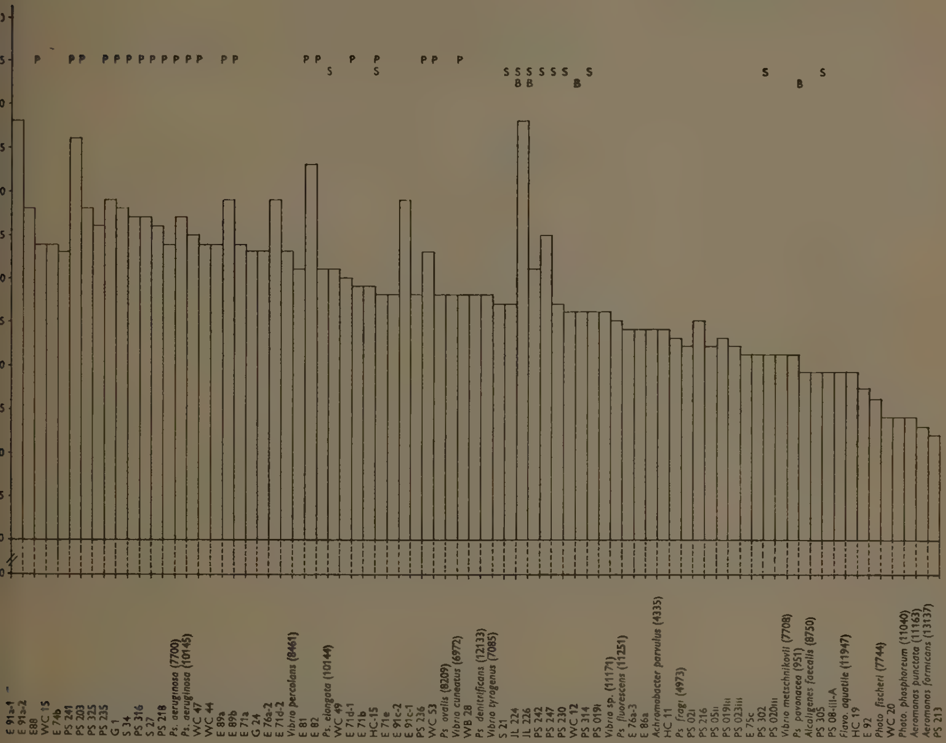

Fig. 2. A diagrammatic representation of the similarities within a group containing 20 strains from type culture collections and 60 unidentified pseudomonads.
P, green pigment formed; B, brown pigment formed; S, sea water required.

A final point which bears on our discussions at this meeting concerns the position of many of the marine strains, which are notable for their lack of reactivity in the usual biochemical and physiological tests. This accounts for their failure to cluster with other organisms in our present analysis, and to bring some order into their classification it seems of prime importance that new tests be sought in which they yield positive reactions. A more thorough investigation of their nutritional requirements, particularly in relation to nitrogen containing compounds, might provide such tests.

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(Symposium on *Pseudomonas* and *Achromobacter*: Contribution relating to Paper II)

COMPUTATION OF SIMILARITIES BETWEEN STRAINS OF *PSEUDOMONAS* AND *ACHROMOBACTER* ISOLATED FROM CHICKEN MEAT

BY MARGARET J. THORNLEY
Low Temperature Research Station, Cambridge

SNEATH'S METHOD of computing similarities between strains of bacteria (Sneath, 1957) was applied to bacteria isolated from chicken meat stored under chill conditions. About 350 strains were identified as either *Pseudomonas* or *Achromobacter* (Thornley, 1960) using mainly the criteria of Shewan (see Shewan, Hobbs & Hodgkiss, 1960), and afterwards subjected to a wide range of tests. From these strains 25 *Achromobacter*, 11 pigmented *Pseudomonas* and 15 nonpigmented *Pseudomonas* were chosen so as to include as much variation in properties as possible. The *Achromobacter* strains were all nonmotile and therefore did not resemble the type species, *A. liquefaciens*. The results for these strains, together with those for 10 *Pseudomonas* strains from other culture collections (see Table 1), were used for the computation of similarities, and the results are shown in Fig. 1.

Table 1. *The numbers and identities of strains from culture collections*

Collection no.	Identity	
NCTC 4755	*Ps. fluorescens*	
NCIB 3756	*Ps. fluorescens*	
NCIB 8296=ATCC 8209	*Ps. ovalis*	
NCIB 8542=ATCC 4973	*Ps. fragi*	
NRRL B 1606	*Ps. geniculata* from Dr. W. C. Haynes, Northern Regional Research Laboratories	
Niven O1 Niven P4	Nonpigmented *Pseudomonas* strains from Dr. C. F. Niven, Jr., American Meat Institute Research Foundation	
MR 28/5(=NCTC 10,038 and ATCC 13,525)	*Ps. fluorescens*. Neotype strain from Dr. M. E. Rhodes	See Rhodes (1959, 1961)
MR 22/1	*Ps. fluorescens* from Dr. M. E. Rhodes	
MR 26/3	*Ps. fluorescens* from Dr. M. E. Rhodes	

All the *Pseudomonas* strains were grouped separately from all the *Achromobacter*, and showed a high degree of similarity, and with few exceptions the pigmented and nonpigmented strains were grouped separately.

The nonpigmented group showed higher S values than the pigmented group, a result differing from that of Liston (1960), who found a converse tendency. This was probably because his nonpigmented group included strains which did not attack glucose (Shewan, Hobbs & Hodgkiss, 1960, groups III or IV) whereas ours did not.

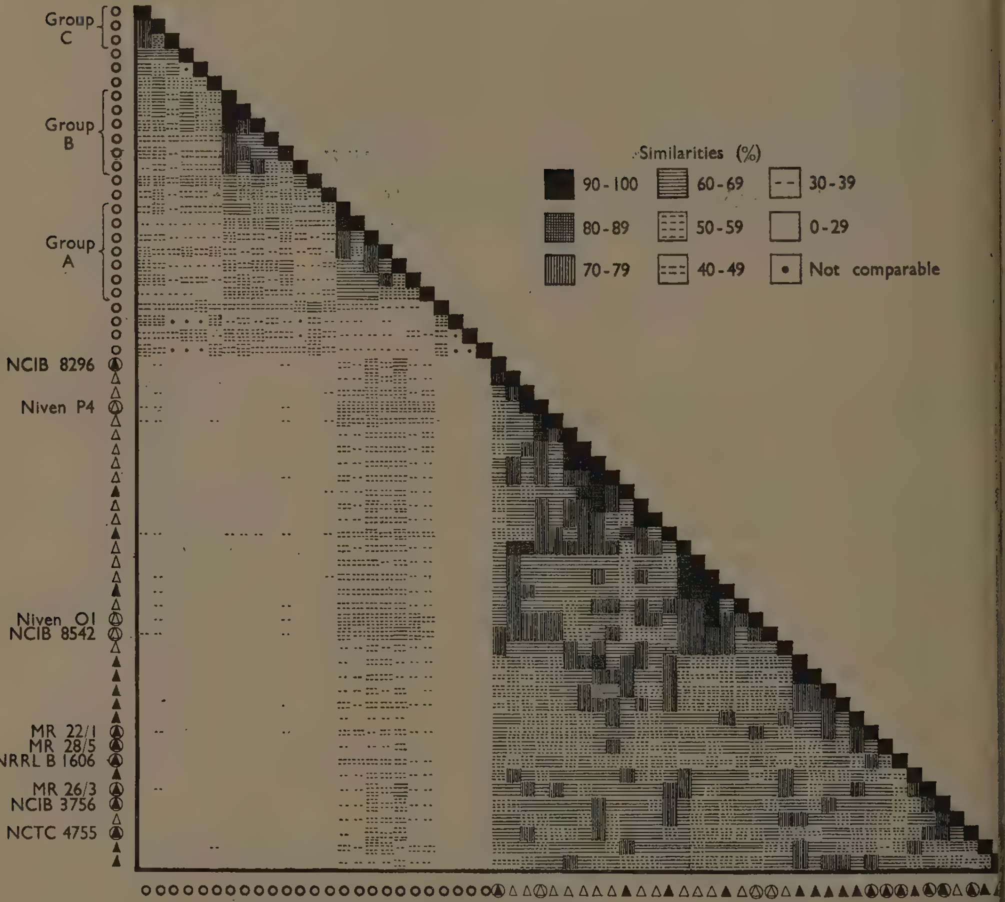

Fig. 1. A diagrammatic representation of the similarities between strains of *Pseudomonas* and *Achromobacter*.
Thick rings, *Achromobacter*; full triangles, pigmented *Pseudomonas*; open triangles, nonpigmented *Pseudomonas*.
Circles around triangle symbols indicate strains from culture collections.

Two *Pseudomonas* strains isolated by Niven from fresh beef produced no fluorescent pigment, and showed close resemblance to our nonpigmented group.

The pigmented group included (see Table 1) NCTC 4755, and NCIB 3756, *Ps. fluorescens*, and NRRL B 1606, *Ps. geniculata*, besides three of the strains of Rhodes (1959), nos 28/5, 22/1 and 26/3, which she considered to be *Ps. fluorescens*. The present grouping supports Rhodes' suggestion that the separation of *Ps. geniculata* from *Ps. fluorescens* is not justified.

The *Achromobacter* strains showed a much lower degree of similarity within the group than *Pseudomonas*, and three subgroups, A, B and C, were indicated.

Group A consisted of strains with an oxidative attack on sugars, usually glucose, galactose, arabinose, xylose and lactose. The first four of these, and sometimes lactose, were also oxidized by *Pseudomonas* strains, and the fact that these strains had a higher similarity to *Pseudomonas* than had the other *Achromobacter* strains is shown in Fig. 1. This group was also sensitive to penicillin and oxytetracycline and, with one exception, oxidase positive.

All the other *Achromobacter* strains failed to oxidise sugars, and showed two rather less clearly defined groups with intermediate strains between them. One of these, group B, contained 7 strains which were sensitive to penicillin and oxytetracycline (with one exception), oxidase positive (with one exception) and unable to grow in Paton's gluconate medium (Paton, 1959). The other, group C, contained 3 strains insensitive to penicillin, sensitive to oxytetracycline, oxidase positive and able to grow in Paton's medium.

It is intended to repeat the procedure with a much larger number of our *Achromobacter* strains, to confirm the existence of these groups. It would also be interesting to extend the work to motile strains, as they would be more similar to the type species of the genus.

It seems clear that our knowledge of *Pseudomonas* is much more extensive than that of *Achromobacter*, while the latter is a more diverse group. It is worth noting that proposals such as that of Liston (1960) to separate genera at an S level of, for instance, 60% would split the *Achromobacter* strains into several genera. It remains to be shown by further work on this group whether such a division would be desirable.

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(*Received* 26 *August*, 1960)

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(Symposium on *Pseudomonas* and *Achromobacter:* Contribution relating to Paper II)

THE INTERPRETATION OF FLAGELLA STAINS

By W. HODGKISS

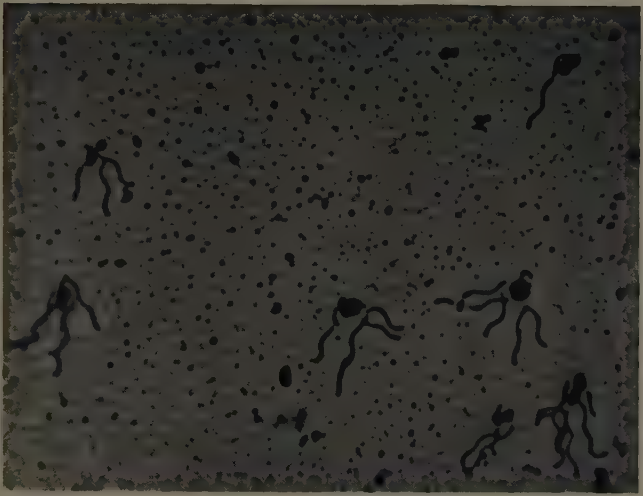
Torry Research Station, Aberdeen

During the discussions, in which criticisms were made of Bergey's *Manual*, Professor Rogosa pointed out that as the *Manual* depends wholly upon published results and hence cannot be more reliable and accurate than those results, it therefore behoves all workers in the field to be accurate in the interpretation of results. My plea is for the use of controls in the interpretation of flagella stains. Flagella staining is a difficult technique, and in addition interpretation of the results is hazardous unless adequate control preparations, of polar and peritrichous organisms, are also stained with the organism under investigation. It is important to remember that a flagellum approximately 200 Å wide has to be considerably 'thickened' in order to be made visible in an optical microscope. Even well stained preparations give relatively inaccurate pictures of the flagellation.

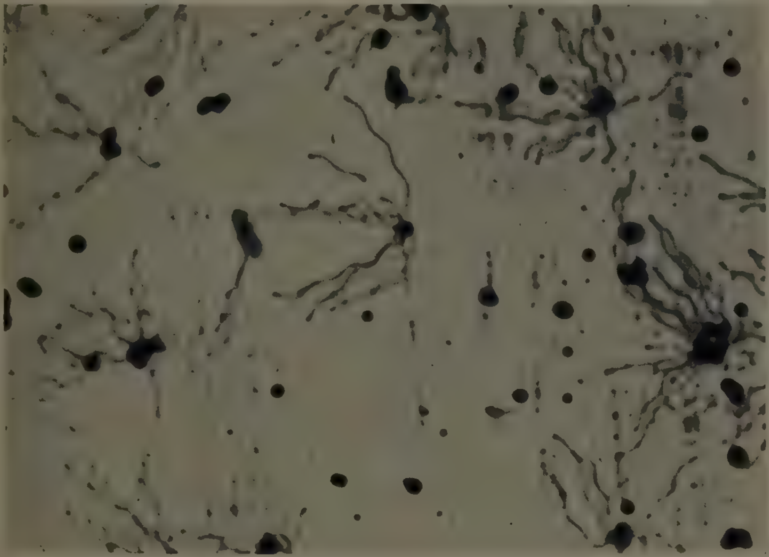
Cook (1958) and Stevenson (1959) both described organisms with the biochemical characteristics of the Enterobacteriaceae but with polar flagella: but these organisms are in fact peritrichously flagellated (Plate 1, a, b and Plate 2, a, b). Certainly cells with one flagellum are seen (and are seen in preparations of *E. coli*!): but the flagellum does not arise from the pole (Plate 2, c and Plate 3, a, b) and it is possible that such a cell may appear to possess a polar flagellum in a stained preparation under an optical microscope. These organisms, then, possess peritrichous flagella; and as their biochemical characteristics, given in the original papers and by Eddy (1960), are typical of the family, they are normal members of the Enterobacteriaceae. Stevenson's organisms are certainly not *Aeromonas* spp., though what constitutes a nonpigmented strain of *Serratia* is outside the scope of this note.

Other cases of the misinterpretation of flagella stains have been reported in the recent literature. The organisms assigned by Asai, Okomura & Tsunoda (1957) to the genus *Kluyvera* have been shown by Eddy (1960) to possess peritrichous flagella and similarly, Shimwell & Carr (1959) have shown that strains of *Gluconobacter* Asai and Shoda, stated by Asai & Shoda (1958) to have polar flagella, are in fact peritrichous.

Passing to the oxidase test of Kovacs (1956), again it is essential to use positive and negative control cultures. Kovacs applied the test to differentiate *Pseudomonas aeruginosa* from members of the Enterobacteriaceae: but we find that a positive reaction is given by all polarly flagellated rods of the genera *Pseudomonas*, *Vibrio* and *Aeromonas* and hence we use the test to differentiate the occasional 'paracolon' from a welter of *Pseudomonas* strains. Flagella stains and electron micrographs have

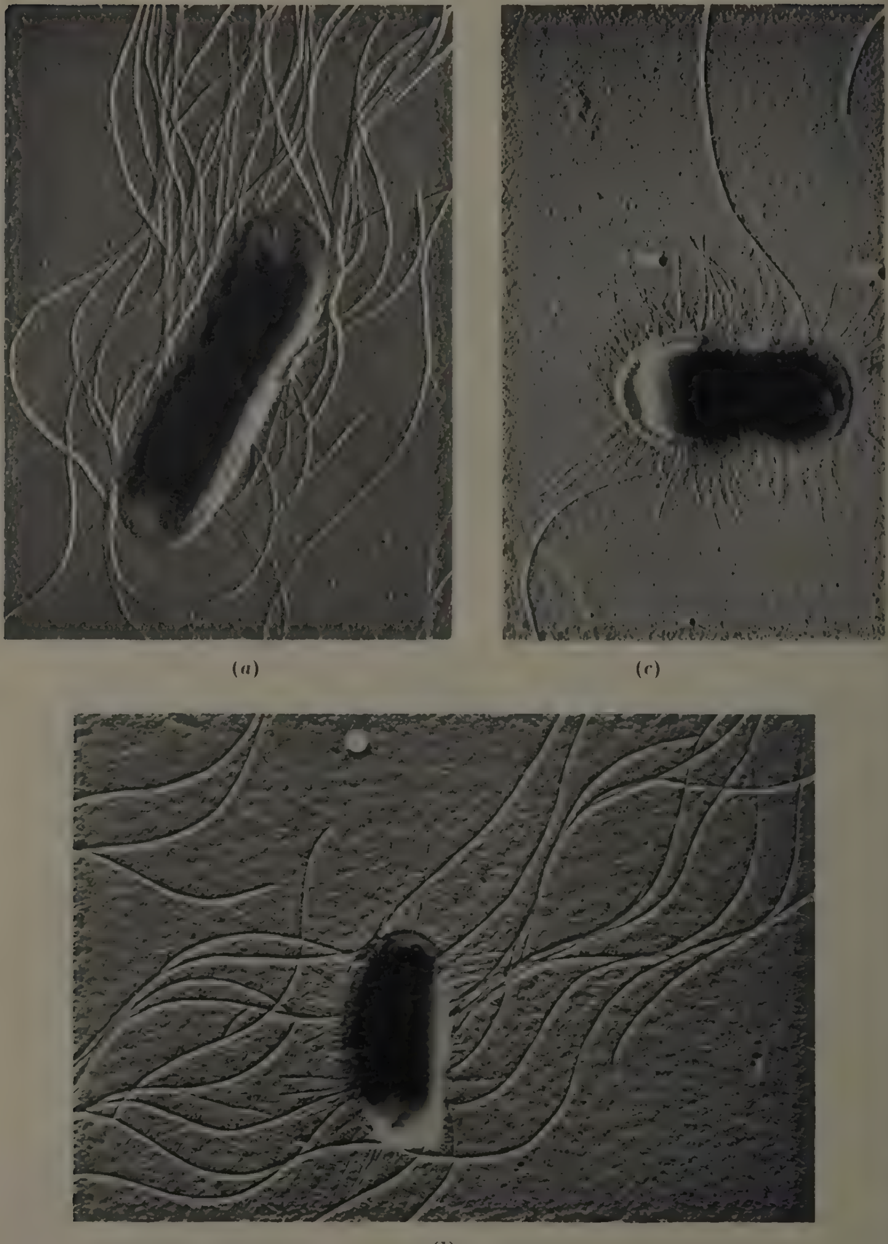


(*a*)



(*b*)

HODGKISS, W. — THE INTERPRETATION OF FLAGELLA STAINS. PLATE 1

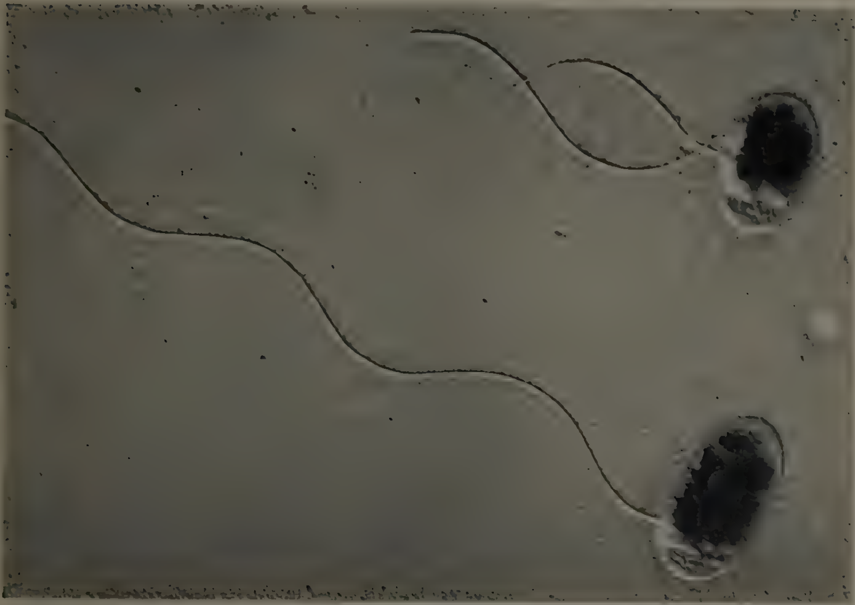


(*a*)

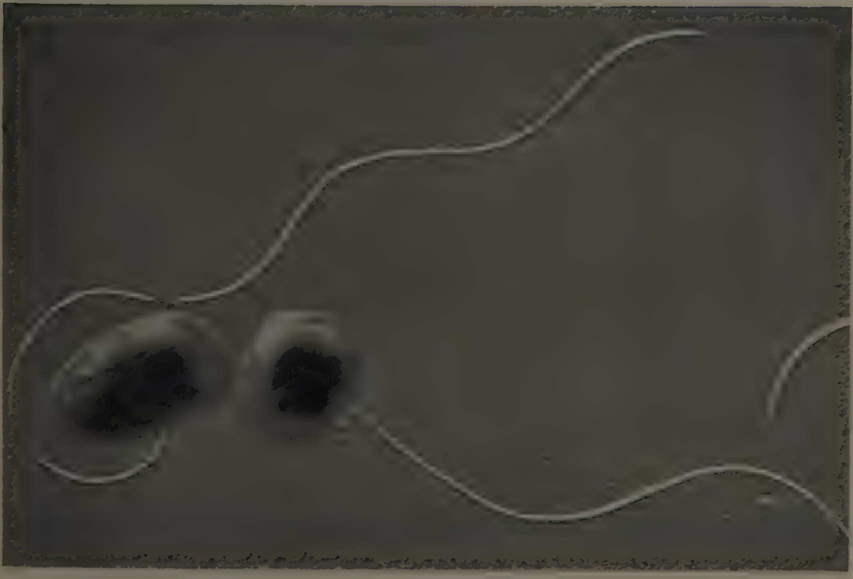
(*c*)

(*b*)

HODGKISS, W. — THE INTERPRETATION OF FLAGELLA STAINS. PLATE 2



(*a*)



(*b*)

Hodgkiss, W.— The Interpretation of Flagella Stains. Plate 3

shown excellent correlation with the oxidase test. All motile Gram-negative peritrichous rods give a negative reaction and polarly flagellated Gram-negative rods, with the possible exception of certain plant pathogens, a positive one. The test has not been applied as a determinative feature for nonmotile organisms (Shewan, Hobbs & Hodgkiss, 1960), but it can be used to differentiate *Aeromonas salmonicida* from *Klebsiella* spp.

This paper was prepared as part of the programme of the Department of Scientific and Industrial Research.

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EXPLANATION OF PLATES

PLATE 1. Flagella stains, modified Casares-Gil method, ×2,000. Cells from 24 hr slope cultures at 20°.
- (a) Culture NCTC 9960 (Cook, 1960).
- (b) Culture SL 10a (Stevenson, 1959).

PLATE 2. Electron micrographs, Au-Pd shadowed, ×20,000.
- (a) and (b) Culture SL 10a (Stevenson, 1959), from 24 hr slopes at 20° and 37° respectively.
- (c) Culture SL 11a (Stevenson, 1959), from a 24 hr slope at 37°.

PLATE 3. (a) and (b) Electron micrographs, Au-Pd shadowed, of culture NCTC 9960 (Cook, 1960) from slope cultures at 20°, ×30,000.

(*Received* 26 *September*, 1960)

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper III)

COMPARATIVE CARBOHYDRATE METABOLISM AND LOCALIZATION OF ENZYMES IN *PSEUDOMONAS* AND RELATED MICRO-ORGANISMS

By J. DE LEY

Laboratory for Microbiology, State University, Ghent, Belgium

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1. Introduction

Pseudomonas is a group of heterotrophic, Gram-negative, asporogenous rod-like bacteria which, if motile, possess polar flagella and are usually strict aerobes. The boundaries of this group are still ill defined and, although about two hundred strains have been given species names, a more logical and natural subdivision into species or biotypes seems to be required. The better and more complete definition of this group and its subdivisions, which is being sought in this Symposium, should not only include ecological, morphological and physiological criteria, but should also lean heavily on the biochemical activities and the enzymic equipment of the organisms. The oxidative capacities of these bacteria and their abilities to use a wide variety of substrates is impressive. This is illustrated, for example, in the thesis of den Dooren de Jong (1926) who showed that *Pseudomonas putida* was able to supply its carbon requirements from 77 compounds out of some 200 examined. The existing information on the biochemistry and enzymology of pseudomonads shows that they are very active in the oxidation of carbohydrates and in the breakdown of aromatic rings. It will probably be in these two fields that the biochemist will be able to supply information and explanations to the taxonomist.

A taxonomical system can only be built up by comparing the properties of many different strains. Up to now the biochemical and enzymic properties of *Pseudomonas* and related aerobic, Gram-negative asporogenous rods have hardly been studied. Only the following superficial physiological properties have occasionally been used for species differentiation: production of fluorescent or other pigments, gelatin liquefaction, temperature optimum, action on milk, cellulose digestion, phosphorescence, production of nitrite from nitrate, acid formation from sucrose, glucose and other sugars, and the Kovacs reaction. Most of the work on the comparative metabolism of the pseudomonads has still to be carried out: e.g. of all the *Pseudomonas* strains described, the intermediary carbohydrate metabolism is well known in only three species, *Ps. fluorescens*, *Ps. aeruginosa* and *Ps. ovalis*.

Since so much remains to be done in this field, it is the aim of this paper to review and discuss briefly the most striking features of the carbohydrate metabolism of the pseudomonads. A general picture of this subject has not yet been published. Much of our knowledge in this field is derived from studies by Doudoroff and his associates on the glucose metabolism of *Pseudomonas saccharophila*. We shall therefore first devote a short discussion to this subject. From a close inspection of the existing data on the carbohydrate metabolism of pseudomonads, we shall postulate a hypothesis on the enzymic anatomy of these organisms. The latter proposal leans heavily on a similar hypothesis for the closely related acetic acid bacteria, which was derived from an extensive study of their comparative carbohydrate metabolism. The latter group of bacteria will therefore be discussed briefly as a model system. Little can be said for the moment on the carbohydrate metabolism of other genera which appear to be closely related to *Pseudomonas*, such as *Xanthomonas*, *Vibrio*, *Agrobacterium*, *Chromobacterium* and *Protaminobacter*. We consider it likely that the general patterns of intermediary carbohydrate metabolism and enzymic anatomy which have been revealed in the acetic acid bacteria and postulated for *Pseudomonas* will occur in these genera also.

2. The Metabolism of *Pseudomonas saccharophila*

By the end of the 1930s the mechanism of glycolysis had been studied in great detail and it was evident that the enzyme system for this pathway was very widespread in nature. Between 1936 and 1955 it was also made clear that glucose-6-phosphate

(G6P) is metabolized by another mechanism, also present in a great variety of cells, which became known as the 'shunt' or the 'hexose monophosphate oxidative cycle'. In 1952–54 Doudoroff and his co-workers discovered that G6P may be metabolized in still a different fashion, now known as the Entner-Doudoroff pathway. This discovery has focused attention on the organism that was used in Doudoroff's investigations, on its many unusual pathways in carbohydrate metabolism and on its unique position in comparative biochemistry.

In 1940 Doudoroff isolated from San Francisco Bay mud a hydrogen bacterium. Like most of these bacteria, it grew both heterotrophically and autotrophically: but it was described under its present name of *Ps. saccharophila* because it exhibited the striking property of growing very well on the disaccharide sucrose, but very poorly on the monosaccharides glucose and fructose (Doudoroff, 1940). The explanation for this behaviour was found in the presence of a new enzyme, called 'sucrose phosphorylase' (Doudoroff, Kaplan & Hassid, 1943). This enzyme has also been found in *Ps. putrefaciens* (Doudoroff, Wiame & Wolochow, 1949) and in *Leuconostoc mesenteroides* (Kagan, Lyatker & Tsvasman, 1942).

The properties of this enzyme have been reviewed on other occasions and a brief summary will suffice here. It is inducible. The apparent reaction is:

$$\text{sucrose}+\text{inorganic phosphate}\rightleftarrows\alpha\text{-D-glucose-1-phosphate}+\text{fructose}$$

The equilibrium constant at pH 6·6 and 30° is 0·053 and not very favourable for the synthesis of the disaccharide. However, the sucrose phosphorylase is really a transglucosidase. It is specific with regard to the glucose moiety of its substrate. It is far less specific with regard to the substituents for the other sucrose component, namely D-fructose. The general reactions are believed to be:

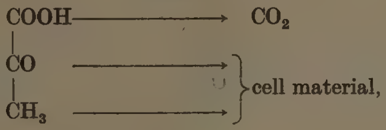
$$\text{sucrose}+\text{enzyme}\rightleftarrows\text{glucosyl-enzyme}+\text{fructose}$$

$$\text{glucosyl-enzyme}+\text{acceptor}\rightleftarrows\text{glucose-acceptor}+\text{enzyme.}$$

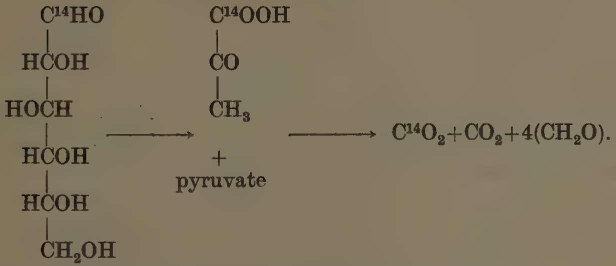
The acceptors are: inorganic phosphate, fructose, L-sorbose, D-xylulose, L-ribulose, D-rhamnulose and L-arabinose. All the ketosugars have to be in the furanose form. L-arabinose reacts because it has the same configuration at C_4, and the disaccharide glucopyranosyl-1,3-L-arabinopyranose contains a 1,3-linkage, which is very rare among polysaccharides.

(a) *The catabolism of glucose: the Entner-Doudoroff pathway*

Not only is the metabolism of sucrose unusual in *Ps. saccharophila*, but glucose and a number of other monosaccharides are also decomposed by a new mechanism, by which two-thirds of the carbon of glucose is assimilated and the rest is released as CO_2 (Doudoroff, 1940; Bernstein, 1944; Whelton & Doudoroff, 1945). At first glance this indicates a C_3–C_3 split; but it cannot happen according to the Embden-Meyerhof pathway, because in experiments in which C_1-labelled glucose was decomposed aerobically the labelled carbon was almost quantitatively recovered as CO_2. This suggests that the shunt was operative. However, this also could not be so, because the addition of dinitrophenol or arsenite resulted in the oxidative formation of two pyruvate molecules from either glucose or gluconate, one of which contained the label in the carboxyl group. This observation eliminated an oxidative decarboxylation of 6-phospho-gluconate (6PG) and showed that the carbon chain of glucose must be split in two before decarboxylation. From previous experiments it was already known that pyruvate is assimilated according to the following general mechanism:


COOH
CO
CH₃
CO₂
cell material,

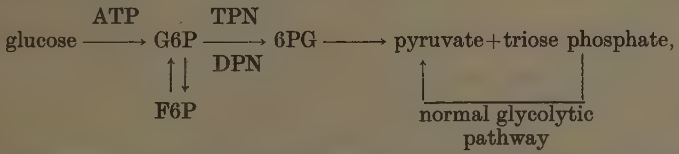
so that


C¹⁴HO
HCOH
HOCH
HCOH
HCOH
CH₂OH
C¹⁴OOH
CO
CH₃
+
pyruvate
C¹⁴O₂+CO₂+4(CH₂O).

In the presence of iodoacetate, the following reaction occurs:

$$1 \text{ glucose} + \tfrac{1}{2}\, O_2 \longrightarrow 1 \text{ pyruvate} + 1 \text{ methylglyoxal}.$$

In cell-free extracts, the enzymes for the following mechanisms were detected (Entner & Doudoroff, 1952):

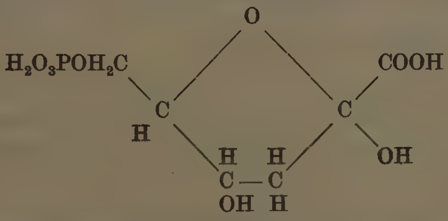


$$\begin{tikzcd}
\text{glucose} \arrow[r, "\text{ATP}"] & \text{G6P} \arrow[r, "\text{TPN}", "\text{DPN}"'] \arrow[d, shift left] & \text{6PG} \arrow[r] & \text{pyruvate} + \text{triose phosphate}, \\
 & \text{F6P} \arrow[u, shift left] & &
\end{tikzcd}$$

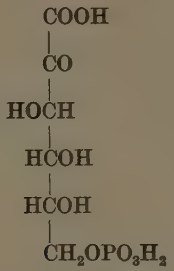
normal glycolytic pathway

where ATP stands for adenosine triphosphate, TPN and DPN for tri- and diphosphopyridine nucleotide and F6P for fructose-6-phosphate.

Entner & Doudoroff postulated that the unknown 2-keto-3-deoxygluconate-6-phosphate was an intermediate, formed by the action of a dehydrase. This compound was indeed isolated two years later by MacGee & Doudoroff (1954). It was prepared by the action of a cell-free gluconate-6-phosphate dehydrase, crystallized as the sodium salt and analyzed. Evidence was also presented that the 2nd and 5th carbon atoms may be joined in a furanoid ring. It has thus the structure


O
H₂O₃POH₂C
COOH
C
H
C
OH
H H
C—C
OH H

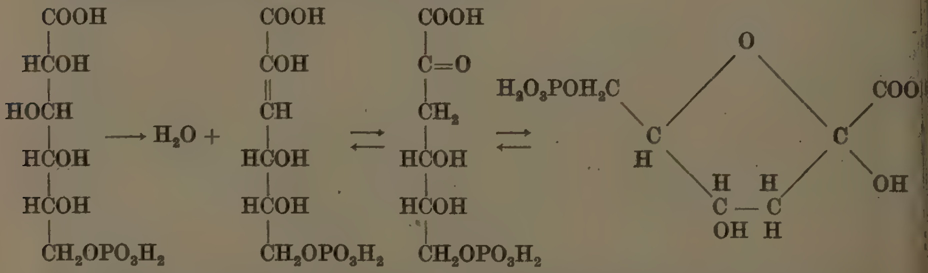
and is structurally closely related to 2-keto-gluconate-6-phosphate, isolated in the same year by De Ley (1954*a*,*b*) with the aid of a 2-ketogluconokinase from *Aerobacter* *cloacae*. The latter intermediate was shown to be chiefly in the open chain form and has the structure



$$\begin{array}{c}\mathrm{COOH}\\ |\\ \mathrm{CO}\\ |\\ \mathrm{HOCH}\\ |\\ \mathrm{HCOH}\\ |\\ \mathrm{HCOH}\\ |\\ \mathrm{CH_2OPO_3H_2}\end{array}$$

It is also of wider occurrence than the MacGee-Doudoroff ester, since it was shown to be formed by numerous bacteria able to decompose 2-ketogluconate. Both esters can be separated by paper chromatography (De Ley, 1955).

These two phosphoric esters have an entirely different meaning for the intermediary metabolism. The MacGee-Doudoroff ester is a real intermediate in the decomposition of glucose-6-phosphate by *Ps. saccharophila* and a limited number of bacterial species. The ester isolated by ourselves provides a route to the hexose monophosphate oxidative cycle, whereby numerous bacteria metabolize 2-ketogluconate after a previous phosphorylation. *Ps. saccharophila* is unable to grow on 2-ketogluconate and also does not form 2-ketogluconate-6-phosphate (De Ley & Doudoroff, unpublished data). The splitting enzyme, 2-keto-3-deoxygluconate-6-phosphate aldolase, was obtained in the cell-free state by MacGee & Doudoroff (1954). The dehydrase and the latter aldolase were later detected by Kovachevich & Wood (1955*a*,*b*) in *Ps. fluorescens* and studied in more detail. The *fluorescens* dehydrase is activated by Fe^{++} and glutathione, whereas the *saccharophila* enzyme is not. The mechanism of dehydration is supposed to be:



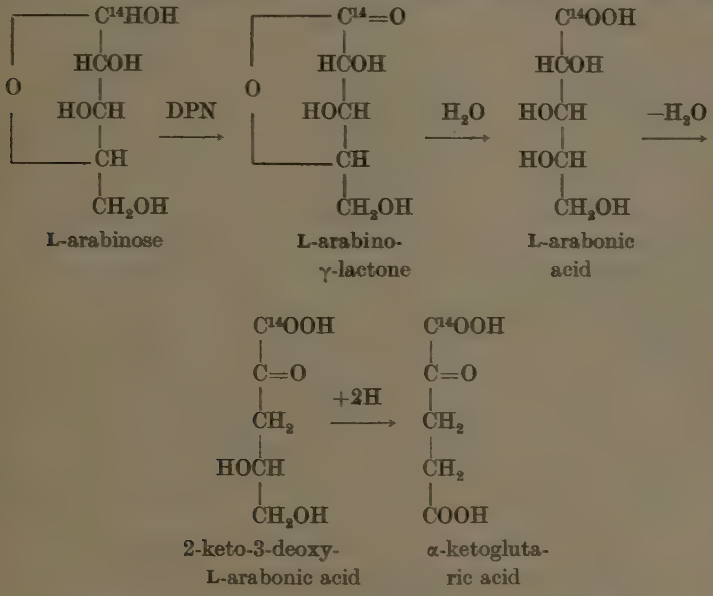
The MacGee-Doudoroff ester resembles deoxyribose-5-phosphate and fructose-1:6-diphosphate, which both have the furanose ring and are split by an aldolase. Cori & Lipmann (1952) have shown that Zwischenferment (G6P-dehydrogenase) oxidizes G6P with the formation of a lactone, according to the sequence

$$\mathrm{G6P} \xrightarrow{\mathrm{TPN}} \text{glucono-}\delta\text{-lactone-6-phosphate} \xrightarrow{H_2O} \mathrm{6PG}.$$

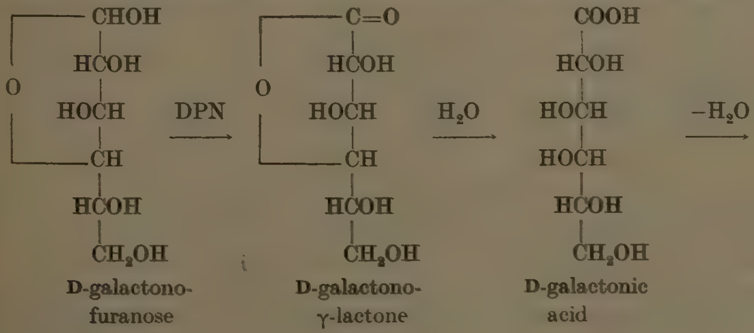
We do not know whether this step has already been looked for in *Ps. saccharophila*. but it is almost sure to happen in this way.

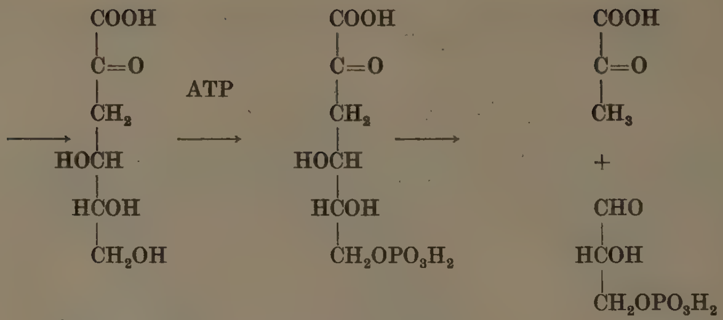
(b) *The catabolism of other sugars*

The study of the metabolism of several other sugars has emphasized the unusual enzymic outfit of *Ps. saccharophila*. Weimberg & Doudoroff (1955) and Weimberg (1959) found that L-arabinose is decomposed according to the following sequence:


$C^{14}HOH$
HCOH
O
HOCH
CH
CH_2OH
L-arabinose
DPN
$C^{14}=O$
HCOH
O
HOCH
CH
CH_2OH
L-arabino-γ-lactone
H_2O
$C^{14}OOH$
HCOH
HOCH
HOCH
CH_2OH
L-arabonic acid
$-H_2O$
$C^{14}OOH$
C=O
CH_2
HOCH
CH_2OH
2-keto-3-deoxy-L-arabonic acid
+2H
$C^{14}OOH$
C=O
CH_2
CH_2
COOH
α-ketoglutaric acid

It could very well be that a similar mechanism for the decomposition of 2:5-diketogluconate exists in *Acetobacter melanogenus* (Datta & Katznelson, 1957). For D-galactose the following pathway was found (De Ley & Doudoroff, 1957):


CHOH
HCOH
O
HOCH
CH
HCOH
CH_2OH
D-galactono-furanose
DPN
C=O
HCOH
O
HOCH
CH
HCOH
CH_2OH
D-galactono-γ-lactone
H_2O
COOH
HCOH
HOCH
HOCH
HCOH
CH_2OH
D-galactonic acid
$-H_2O$

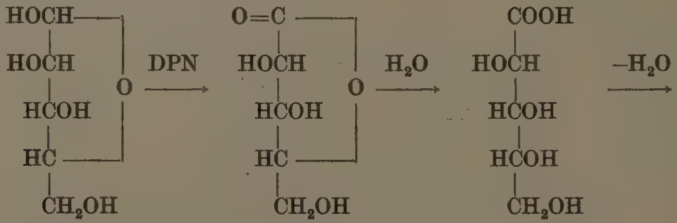


2-keto-3-deoxy-D-galactonic acid

2-keto-3-deoxy-D-galactonate-6-phosphate (not isolated)

2-Keto-3-deoxy-D-galactonic acid, another new intermediate, was enzymically prepared, purified, analyzed and its structure proved by chemical synthesis.

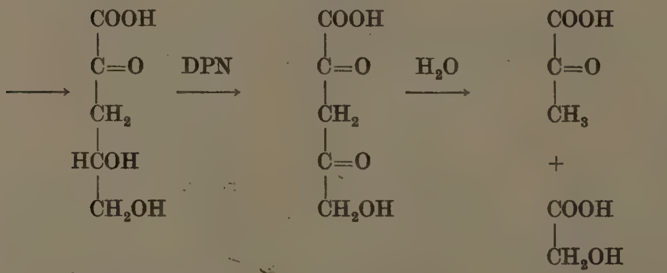
In the metabolism of D-arabinose another new intermediate was found, namely 2-keto-3-deoxy-D-arabonic acid. The complete reaction sequence is as follows (Palleroni & Doudoroff, 1956*a*, 1957):



D-arabino-furanose

D-arabino-γ-lactone

D-arabonic acid



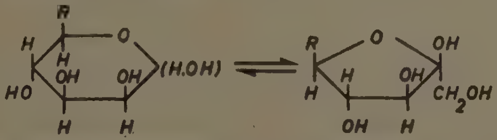
2-keto-3-deoxy-D-arabonic acid

(not isolated)

pyruvic acid and glycollic acid

It is truly remarkable that glucose, galactose, D-arabinose and L-arabinose are decomposed by a set of enzymes of similar activity but different nature. These enzymes are: a dehydrogenase, either TPN- or DPN-linked, which causes the formation of a lactone; a delactonase, with the formation of an open-chain acid; a dehydrase, with the formation of a 2-keto-3-deoxy compound; and an aldolase, with the formation of pyruvate from the upper part of the original sugar molecule, the lower part being transformed into either glyceraldehyde-3-phosphate or glycollic acid.

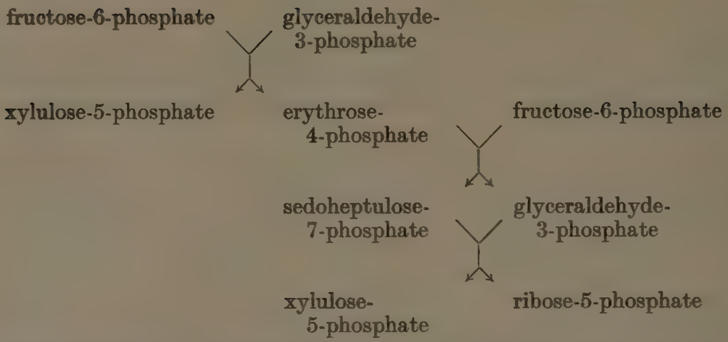
It must be stressed that whereas the enzymes for the metabolism of glucose are constitutive, those for the other systems are inducible. Even cells grown autotrophically on CO_2, H_2, O_2 and mineral salts (De Ley & Doudoroff, unpublished data) still contain the whole set of glucose metabolizing enzymes. The difference between glucose and galactose metabolism consists in the phosphorylative step, which precedes the main pathway in the case of glucose and is the last step in the sequence in the case of galactose. Both arabinoses are metabolized without the participation of phosphorylated intermediates. The analogy stops here, for the other monosaccharides and derivatives are metabolized by a different pathway. Gluconate is not dehydrated with the formation of 2-keto-3-deoxygluconate, but has only one way to enter the metabolism: by an inducible kinase (De Ley & Doudoroff, unpublished data). Fructose is another compound which is of interest, because it is an end product of both invertase and sucrose phosphorylase action. Fructose can be transformed by way of either a kinase or a mannose isomerase. A fructose adapted mutant contains a specific fructokinase, yielding fructose-6-phosphate, which is then transformed into G6P and disappears by way of the Entner-Doudoroff pathway (Palleroni, Contopoulou & Doudoroff, 1956). These cells also contain a new enzyme, 'mannose isomerase' (Palleroni, Contopoulou & Doudoroff, 1956; Palleroni & Doudoroff, 1956*b*), the specificity of which is of some interest for enzymology. It rather rigidly requires a definite structural configuration of the first 5 carbon atoms of the substrates, and the reaction may be generalized as follows:



where R can be any of the following radicals: $-H$ (in D-lyxose), $-CH_3$ (in D-rhamnose), $-CH_2OH$ (in D-mannose) or $-CHOH.CH_2OH$ (in sedoheptulose). It cannot be either $-COOH$ (in mannuronic acid) or a phosphorylated carbinol group (as in mannose-6-phosphate). The latter compound is formed by a kinase and is apparently not metabolized further. Just as in the case of glucose-1-phosphate, this is either a dead end in the metabolism or the enzymes have completely escaped the investigators.

(c) *The formation of pentoses*

We can now turn our attention to another problem: how does *Ps. saccharophila*, grown on a hexose or on sucrose, make its pentoses for the synthesis of nucleic acids? In numerous attempts, De Ley & Doudoroff (unpublished results) have failed to find a 6PG-dehydrogenase which would constitute the most direct way to ribose-5-phosphate. Another way by which this can happen is the reversal of the transketolase-transaldolase system according to the equations:



The xylulose-5-phosphate is ultimately also converted into ribose-5-phosphate (R5P) by way of ribulose-5-phosphate.

It is known that the equilibrium of these reactions is strongly in favour of the hexoses. In *Ps. saccharophila* exactly the same system is present (De Ley & Doudoroff, unpublished data). By incubating R5P with cell-free extracts, the pentose phosphate disappears completely; this is accompanied by a temporary formation of sedoheptulose phosphate and a final accumulation of F6P and G6P, as required by the equilibrium of the phosphohexo-isomerase reaction. Starting from the opposite direction with G6P and a trace of triose phosphate, the same equilibrium of G6P, F6P and a trace of sedoheptulose is obtained. No pentose is of course detected. The direction of the equilibrium is in fact a very happy event. Indeed, it will only be displaced when the growing cells draw off the pentose phosphate for the synthesis of its nucleoproteins and ribose containing cofactors. Thus, no pentose can continuously leak from the G6P pool and be lost as useless end product. Our unpublished results were later confirmed by Bernstein & Fossit (1960). With glucose-2-C^{14}, 94% of the label was recovered as ribose-2-C^{14} and 89% in deoxyribose-2-C^{14}. With glucose-1-C^{14}, the C_1 of ribose had 92% of the label and with glucose-6-C^{14} it was distributed between C_5 (74%) and C_1 (18%). This was in agreement with the opinion that most of the ribose was formed by the transketolase and transaldolase mechanism and desoxyribose from ribose or a common intermediate. If R5P is made according to the regular hexose monophosphate oxidative cycle from glucose, the total overall equation reads

$$\text{glucose} + \text{ATP} + 2\ \text{TPN}^+ + H_2O \longrightarrow \text{R5P} + CO_2 + 2\ \text{TPNH} + 2\ H^+ + \text{ADP}.$$

In the case of *Ps. saccharophila* it becomes

$$\text{glucose} + \text{ATP} + 1/3\ \text{TPN}^+ \longrightarrow \text{R5P} + 1/3\ \text{pyruvate} + 1/3\ \text{TPNH} + 1/3\ H^+ + \text{ADP}.$$

Both mechanisms have their advantages. The energy is present potentially in TPNH and will either be lost as heat or converted into ATP when TPNH is finally oxidized by O_2. In *Ps. saccharophila* only one-sixth of the direct energy, accompanying forcibly the R5P formation, is available as TPNH; the rest is potentially present in pyruvate, which can still be used for the synthesis of cell material. It is remarkable that *Ps. saccharophila* has all the enzymes of the shunt except the 6PG dehydrogenase, and this might very well be the reason why the Entner-Doudoroff mechanism is required at all. Indeed, ribose is essential for the synthesis of cell material, and since *Ps. saccharophila* cannot make its ribose from the regular decarboxylation of 6PG it uses the system as described above, for which it requires glyceraldehyde-3-phosphate; and this is made by the Entner-Doudoroff mechanism. Of course, if a bacterium which relied only on the hexose monophosphate cycle for pentose synthesis should lose the 6PG dehydrogenase by mutation, this would be a lethal mutation. On the other hand, if the Entner-Doudoroff mechanism is present, the bacterium can dispense with the 6PG-dehydrogenase, which is no longer absolutely necessary.

(d) *Conclusions*

For comparative biochemistry, the Entner-Doudoroff mechanism has an important meaning, because so far it has been detected only in a few micro-organisms and is decidedly absent 'in toto' in others. It is present mostly in strictly aerobic, Gram-negative bacteria. The importance of this mechanism in *Pseudomonas* proper will be discussed below. Several other pseudomonads, and organisms of the genera *Xanthomonas*, *Agrobacterium*, *Rhizobium* and *Azotobacter* (see below) also contain the enzymes of this system. The anaerobic *Zymomonas mobilis* (*Pseudomonas lindneri*, the well known alcohol producing bacterium, isolated from the fermenting sap or 'pulque' of the agave in Mexico) appears to contain only the Entner-Doudoroff pathway (Wang, Gilmour & Cheldelin, 1958). It is surprising that the completely unrelated species *Streptococcus faecalis* can also use that pathway (Sokatch & Gunsalus, 1957), but on the other hand it is not present in several corynebacteria, in species of the genera *Proteus*, *Leuconostoc*, *Lactobacillus* and *Microbacterium*, or in brewer's yeast. There are no reports yet on the presence of this system in higher plants and animals, but from the available data on the distribution of the carbon atoms from labelled glucose during its catabolism in the liver and brain, etc., it is clear that glycolysis and the shunt are the main, if not the only, mechanisms operative. The presence of the Entner-Doudoroff pathway in many of the above mentioned bacteria is very interesting, because it is already known that several of them are closely related physiologically and in other ways, and this now emphasizes the relationship yet again. It also suggests that on closer examination organisms of the genera *Vibrio*, *Gluconobacter*, *Protaminobacter*, *Achromobacter* and *Spirillum* might prove to contain this system. One would almost expect them to, and it looks also as if these considerations strengthen the temptation to suggest that, from an evolutionary point of view, these bacteria belong to a different branch. The existence of the Entner-Doudoroff mechanism shows that the two classical types of carbohydrate metabolism are by no means the only ones, and that nature has devised other ways of obtaining energy from carbohydrates. Possibly this will not be the last to be discovered.

3. THE COMPARATIVE CARBOHYDRATE CATABOLISM OF ACETIC ACID BACTERIA

The acetic acid bacteria form a group of obligately aerobic, Gram-negative rod shaped bacteria which share the common property of oxidizing ethanol to acetic acid in the pH range 4–7·5. This sets them apart from the other Gram-negative, asporogenous, strictly aerobic rods. Stanier (1947) pointed out that whereas some

Pseudomonas spp. are able to carry out the same oxidation, they are less acid-resistant than the acetic acid bacteria since they die at a pH below 5. The physiological and biochemical properties of the different representatives of the acetic acid bacteria vary widely. On the one hand, there are strains with a very limited equipment of enzymes, which are able to oxidize only lactate, ethanol and some Krebs cycle intermediates, e.g. *Acetobacter peroxydans*. On the other hand, there are bacteria with a very wide range of enzymic possibilities which are able to oxidize many carbohydrates and derivatives incompletely, e.g., *Gluconobacter liquefaciens*. They oxidize, for example, polyalcohols to the corresponding ketosugars, hexoses and pentoses to hexonates and pentonates and gluconate to 2- and 5-ketogluconate.

Frateur (1950) proposed an excellent classification of these bacteria, based on their physiological and biochemical properties. In this system the existing strains were arranged in four groups: peroxydans, oxydans, mesoxydans and suboxydans. A brief summary of the characteristics of these groups is given in Table 1. Up to that time about forty different species and varieties had been described. Frateur simplified the situation considerably and reduced the species to ten. Thus the separate species *Acetobacter rancens*, *A. pasteurianus* and *A. kützingianus* were classified together as one species, *A. rancens*. Our enzymic analysis has completely substantiated Frateur's proposal. There is now a tendency to separate the acetic acid bacteria into two genera based on flagellation, the polar organisms into one, for which the names *Gluconobacter* or *Acetomonas* have been proposed, and the peritrichous organisms into another, with the name of *Acetobacter*. Asai (1934, 1935) had already proposed such a division, basing his views on the physiological properties. He also suggested the name *Gluconobacter*. This genus was again studied extensively by Asai & Shoda (1958). Leifson (1954) discovered that the bacteria of the suboxydans group of Frateur were polarly flagellate and proposed the name *Acetomonas* for them. The latter strains can easily be included in the genus *Gluconobacter* and for historical reasons we shall retain the latter name in the present discussion. The genus *Gluconobacter* (Asai) Asai and Shoda needs some redefinition (we omit here Asai's strains which do not oxidize ethanol to acetate and obviously are pseudomonads), as it includes the polarly flagellate or nonflagellate bacteria which oxidize glucose to

Table 1. *The classification and properties of the acetic acid bacteria, according to different authors*

Group (Frateur)	Criteria* according to Frateur					Flagella* (Leifson)	Genera	
	Catalase	Acid from glucose	Ketogenesis†	Acetate oxidation	CO_2 from lactate		Asai	Leifson
Peroxydans	−	−	−	+	+	peri or −	*Acetobacter*	*Acetobacter*
Oxydans	+	+‡	−	+	+	peri or −		
Mesoxydans	+	+	weak	+	+	peri or −		
	+	+	+	+	+	polar or −	*Gluconobacter*	*Acetomonas*
Suboxydans	+	+	+	−	−	polar or −		

* +, positive; −, negative or absent. † Mannitol to fructose; glycerol to dihydroxyacetone; gluconate to 5-ketogluconate.

‡ The species *ascendens* is negative.

gluconate in considerable amounts, oxidize ethanol weakly and slowly to acetic acid, usually do not oxidize acetate and lactate to CO_2 and water, and produce γ-pyrones. *Acetobacter* (Beijerinck) Leifson includes the strains of the mesoxydans, oxydans and peroxydans groups of Frateur. It could very well be that *Gluconobacter* is related phylogenetically to *Pseudomonas* and *Acetobacter* to *Achromobacter*.

(a) *Comparative carbohydrate metabolism*

A study of the literature in conjunction with the results obtained in our laboratory shows that the acetic acid bacteria contain two sets of enzymes: a core of enzymes, present in all the strains, and enzymes which occur in some strains and not in others and are often responsible for the species differences.

The enzymes which are present in all the strains are responsible for the hexose monophosphate oxidative cycle or shunt and the oxidation of lactate and ethanol to acetate.

The enzymes of the hexose monophosphate oxidative cycle or shunt were demonstrated by Hauge, King & Cheldelin (1955), Kitos *et al.* (1958) and by Gromet, Schramm & Hestrin (1957) in *G. suboxydans* and *A. xylinum*, respectively. Stouthamer (1960) detected the same mechanism in *G. liquefaciens*. Cell-free extracts of *A. peroxydans* oxidize the phosphoric ester intermediates of this cycle (De Ley, 1958). In many strains of *Acetobacter* and *Gluconobacter* we have detected different enzymes of the shunt mechanism.

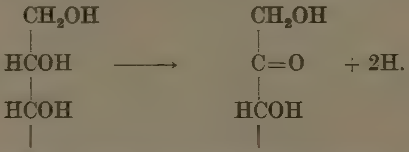
The prototype of the mechanism of the oxidation of lactate and ethanol to acetate was studied by De Ley & Schell (1959) in *A. peroxydans*, which contains two sets of enzymes for these conversions. First, there are ultramicroscopic particles which contain the complete enzyme equipment for the oxidation of D- and L-lactate to acetate, CO_2 and water, as well as for the oxidation of ethanol to acetate and water. These particles are self-contained units which bear all the enzymes and cofactors for these conversions. We shall return below to their true nature. Second, in the cytoplasm there are soluble enzymes which carry out identical chemical reactions with both lactate isomers and with ethanol. However, these enzymes are of a different nature. A summary of the activities of both the particle-linked and the soluble enzymes in *A. peroxydans* is given in Fig. 2. Once the metabolism of D- and L-lactate and ethanol was known in *A. peroxydans*, Schell (1960) studied it in *A. aceti*, *A. rancens*, *A. xylinum*, *G. suboxydans* and *G. liquefaciens* and found exactly the same system. We found that 45 strains of acetic acid bacteria, without exception, oxidized both lactate isomers to acetate, probably always by the same mechanism.

There are seven known enzymes and enzyme systems which are irregularly distributed over the strains of the acetic acid bacteria, as follows.

(i) The Krebs cycle is probably present in all species which 'overoxidize' acetic acid. The presence of these enzymes was detected in *A. pasteurianus* by King, Kawasake & Cheldelin (1956), in *A. aceti* by Rao (1955) and in *G. liquefaciens* by Stouthamer (1960). The strains of *Gluconobacter* which are unable to oxidize acetic acid (the suboxydans group of Frateur) probably lack some of the enzymes of this cycle. Rao (1955) showed that *G. suboxydans* contains only fumarase and aconitase. The other enzymes are absent.

(ii) Most strains oxidize hexoses to hexonates, pentoses to pentonates and gluconate to 2-ketogluconate. Glucose is oxidized to gluconate by all strains except *A. ascendens*, *A. paradoxus* and *A. peroxydans*. Gluconate is oxidized rapidly to 2-ketogluconate by many strains of *Gluconobacter* and by the mesoxydans group of *Acetobacter*. The oxydans group of *Acetobacter* produces this substance only slowly (Frateur, Simonart & Coulon, 1954). Nearly all the strains, apart from a few representatives of the oxydans group and those of the peroxydans group, oxidize mannose to mannonic acid and galactose to galactonic acid. None of the pentoses is oxidized beyond the pentonate stage. We have shown (De Ley & Dochy, 1960*a*) that these oxidations occur on the cytoplasmic membrane of the organisms.

(iii) The ketogenic properties of the mesoxydans group of *Acetobacter* and *Gluconobacter* are summarized by the well known rule of Bertrand, so that the following oxidation occurs:



$$\begin{array}{c} CH_2OH \\ | \\ HCOH \\ | \\ HCOH \\ | \end{array} \longrightarrow \begin{array}{c} CH_2OH \\ | \\ C{=}O \\ | \\ HCOH \\ | \end{array} + 2H.$$

Thus the oxidation of many polyols (e.g. mannitol to fructose, sorbitol to sorbose or fructose, perseitol to perseulose, glycerol to dihydroxyacetone, etc.), of 2:3-butanediol to acetoin and of gluconate to 5-ketogluconate proceed according to this rule.

(iv) The formation of acetoin from pyruvate occurs in many strains. In *A. rancens* and closely related strains these enzymes are particularly active and up to 74% of DL-lactate is converted into acetoin (De Ley, 1959). We found that two mechanisms are involved, both of them effected by soluble enzymes according to the reactions

$$2\ \text{pyruvate} + 2\ \text{ThPP} \longrightarrow 2\ \text{`aldehyde-ThPP'} + 2\ CO_2$$

$$\text{`aldehyde-ThPP'} \longrightarrow \text{acetaldehyde} + \text{ThPP}$$

$$\text{`aldehyde-ThPP'} + \text{acetaldehyde} \longrightarrow \text{acetoin} + \text{ThPP}$$

and

$$\text{`aldehyde-ThPP'} + \text{pyruvate} \longrightarrow \alpha\text{-acetolactate} + \text{ThPP}$$

$$\alpha\text{-acetolactate} \longrightarrow \text{acetoin} + CO_2,$$

where ThPP stands for thiamine pyrophosphate.

(v) Peroxydases were first studied by Wieland & Pistor (1936, 1938) in *A. peroxydans* and were later studied extensively in our laboratory. These enzymes use D- and L-lactate, acetaldehyde, primary alcohols, and *p*-,*o*- and dimethyl-*p*-phenylenediamine as substrates. They are weakly present in most acetic acid bacteria, but particularly active in the peroxydans group of *Acetobacter*. They are located on the cytoplasmic membrane of the cells (De Ley, unpublished data).

(vi) Catalase is not present in the peroxydans group of *Acetobacter* (Frateur, 1950) but present in all other strains. Occasionally a strain has been found which did not belong in the peroxydans group and nevertheless lacked catalase, such as *A. xylinum* NCIB 8623, *A. rancens* and *A. ascendens* cat–.

(vii) Polysaccharides are formed by several species. *A. xylinum* is notorious for making cellulose. So do *A. xylinoides*, *A. orleanense*, *A. acetigenum* and *A. estunensis*

(Carr, 1958). *G. capsulatus* produces dextran (Hehre & Hamilton, 1951) and *A. pasteurianus* makes a starch-like polysaccharide. The ability to make polysaccharides is easily lost.

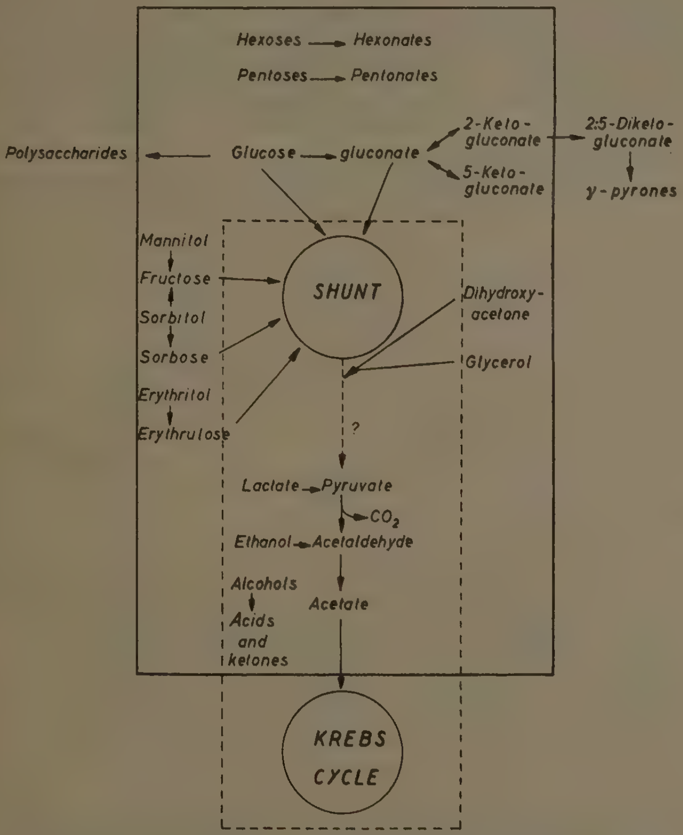

Hexoses
Hexonates
Pentoses
Pentonates
Polysaccharides
Glucose
gluconate
2-Keto-gluconate
5-Keto-gluconate
2:5-Diketo-gluconate
γ-pyrones
Mannitol
Fructose
Sorbitol
Sorbose
Erythritol
Erythrulose
SHUNT
Dihydroxy-acetone
Glycerol
?
Lactate
Pyruvate
CO_2
Ethanol
Acetaldehyde
Alcohols
Acids and ketones
Acetate
KREBS CYCLE

Fig. 1. A simplified metabolic map for the acetic acid bacteria. The overall picture represents the main part of the metabolism of *G. liquefaciens.* Although the latter strain is not known to make polysaccharides, this property has been included because it is common to many strains. Two typical extreme divergencies are outlined by the continuous lines (*G. suboxydans*) and the broken lines (*A. peroxydans*). From De Ley (1961).

By using the above criteria as a guide we have studied the enzymic constitution of many strains and have established a general metabolic map for the acetic acid bacteria which is represented in Fig. 1. *Gluconobacter liquefaciens* strain 20 has undoubtedly the richest enzymic outfit of all the strains investigated and *A. peroxydans* the poorest.

(b) *The enzymic anatomy*

A metabolic map of a group of bacteria is essential for a better knowledge of their mutual relationships. However, this picture can be still more refined if the location of the enzymes in the cell can be discovered. That all the enzymes are not distributed at random through the cytoplasm can be seen from our results on the dual system for the oxidation of lactate and ethanol, which are summarized in Fig. 2. The oxidation of gluconate to 2-ketogluconate affords another instance: here also there are two systems, one soluble and the other particle-linked (De Ley & Stouthamer, 1959), as shown in Fig. 3. The same situation also exists with the oxidation of butanediols (Kersters & De Ley, unpublished results). These ultramicroscopic particles, which bear a number of dehydrogenases and the complete cytochrome system, have been called oxidosomes by De Ley (1960). A search for these oxidosomes revealed that they do not exist as such in the cell; they are not free-floating in the cytoplasm, but are fragments of the cytoplasmic membrane, which arise during the mechanical or ultrasonic breakage of the cell (De Ley & Dochy, 1960*a*). *Gluconobacter liquefaciens* cells were converted into 'protoplasts' by a method involving digestion of the cell wall with human serum. In a hypotonic medium these 'protoplasts' undergo lysis

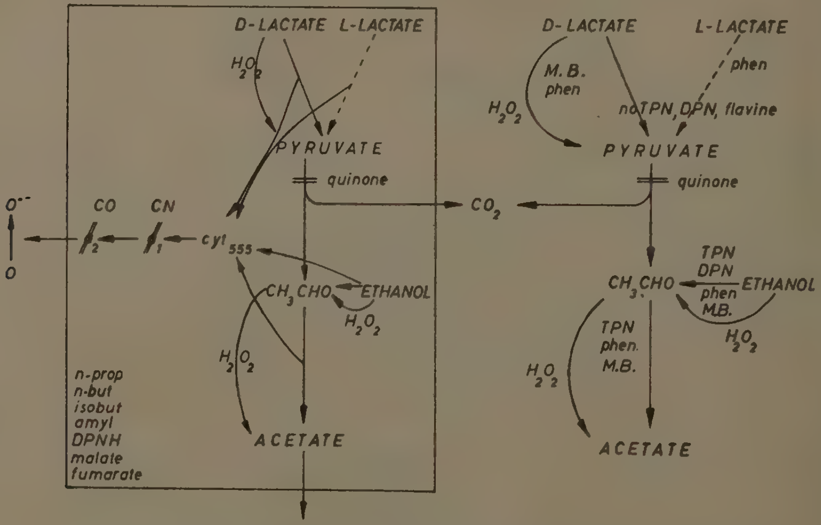

D-LACTATE
L-LACTATE
H_2O_2
PYRUVATE
quinone
O··
O
CO
CN
a_2
a_1
cyt 555
CH_3CHO
ETHANOL
H_2O_2
H_2O_2
n-prop
n-but
isobut
amyl
DPNH
malate
fumarate
ACETATE
CO_2
D-LACTATE
L-LACTATE
M.B.
phen
phen
H_2O_2
no TPN, DPN, flavine
PYRUVATE
quinone
TPN
DPN
phen
M.B.
CH_3CHO
ETHANOL
H_2O_2
TPN
phen.
M.B.
H_2O_2
ACETATE

Fig. 2. The two pathways for the formation of acetate from either lactate or ethanol by enzymes from *Acetobacter peroxydans*. The rectangle represents an ultramicroscopic particle (oxidosome), the enclosed reactions being linked to it. The other reactions are carried out by soluble enzymes in the cytoplasm. Summarized from the results of De Ley & Schell (1959). Explanations of abbreviations are given in Appendix 3.

and the ghosts (cytoplasmic membranes) can be isolated. The purified ghosts are able to oxidize a number of substrates, such as glucose, gluconate, lactate, ethanol, etc., without the addition of cofactors, and they contain the same enzymes as the oxidosomes. Further, by ultrasonic breakage they are fragmented into ultramicroscopic particles which behave physically and enzymically in the same way as the oxidosomes. A second proof of the true nature and origin of the oxidosomes was obtained from a study of the kinetics of the ultrasonic disruption of the cells (De Ley & Dochy, 1960*b*), which showed that the oxidosomes originated from the solid cell

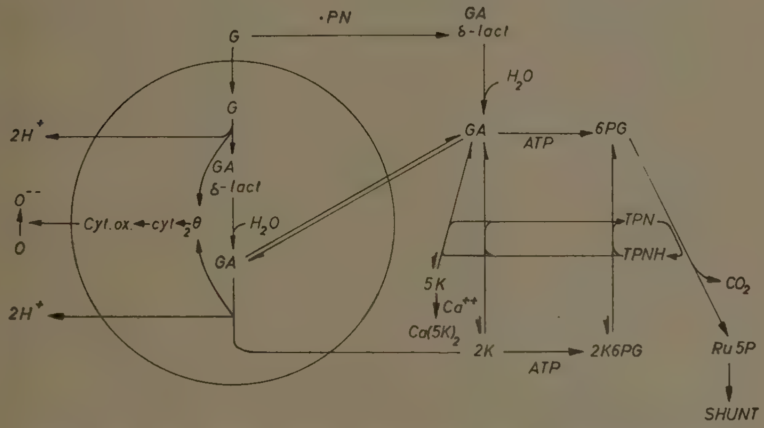


Fig. 3. The two pathways for the oxidation of glucose by *Gluconobacter* and some strains of the mesoxydans group of *Acetobacter*. The circle represents an ultramicroscopic particle (oxidosome) which carries the enzymes for the reactions included therein. The other reactions are carried out by soluble enzymes in the cytoplasm. Summarized from the results of De Ley & Stouthamer (1959). Explanations of abbreviations are given in Appendix 3.

envelope or 'hull'. Stouthamer (1960) independently also concluded that the oxidase-bearing particles originate from the cytoplasmic membrane. It becomes therefore possible to construct a picture of the enzymic anatomy of the cell of *G. liquefaciens* (Fig. 4). Although most other species of acetic acid bacteria have not been converted into protoplasts, so that direct evidence of origin is lacking, oxidosomes have been isolated from all the strains investigated. Intermittent ultrasonic disruption of several strains has always shown that the oxidosomes were derived from the 'hull'. Other acetic acid bacteria thus appear to be constructed essentially in the same way as *G. liquefaciens*, but they are considerably simpler. The differences consist mostly in the enzymic activity of the cytoplasmic membrane. The typical behaviour of the acetic acid bacteria is mostly directed by the enzymic outfit of their cytoplasmic membranes. Species differentiation also is in many cases determined by that structure.

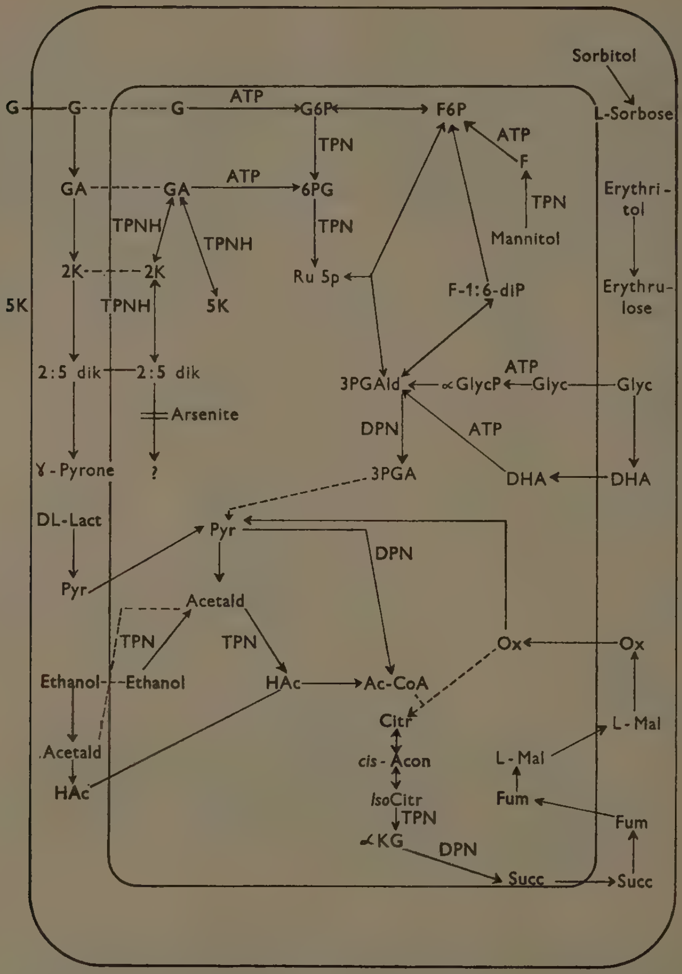

Sorbitol
G
G
G
ATP
G6P
F6P
L-Sorbose
TPN
ATP
F
GA
GA
ATP
6PG
TPN
Erythri-tol
TPNH
TPNH
TPN
Mannitol
2K
2K
Ru 5p
F-1:6-diP
Erythru-lose
5K
TPNH
5K
2:5 dik
2:5 dik
ATP
3PGAld
∝GlycP
Glyc
Glyc
Arsenite
DPN
ATP
Ɣ-Pyrone
?
3PGA
DHA
DHA
DL-Lact
Pyr
DPN
Pyr
Acetald
TPN
TPN
Ox
Ox
Ethanol
Ethanol
HAc
Ac-CoA
Citr
L-Mal
Acetald
cis-Acon
L-Mal
HAc
IsoCitr
Fum
TPN
Fum
∝KG
DPN
Succ
Succ

Fig. 4. The enzymic anatomy of *G. liquefaciens*: a suggested map of the enzymes involved in the carbohydrate metabolism. The outer strip represents the cytoplasmic membrane and includes the enzymes linked to it. The inner part represents the cytoplasm with the soluble enzymes. (From Stouthamer, 1960). Explanations of abbreviations are given in Appendix 3.

(c) *The natural classification*

A natural classification of bacteria must be based on Adansonian principles, where morphological, physiological, biochemical, enzymic, ecological, immunological and other properties have equal value. Within a group of closely related organisms this otherwise complex analysis is considerably simplified because only the points of divergence have to be taken into account. Acetic acid bacteria are particularly well suited for this kind of investigation, because their species differentiation is based mainly on their carbohydrate metabolism. At first glance the differences between the strains are considerable and seem fully to justify the existing differentiation into species: e.g. the difference between *A. suboxydans* and *G. melanogenus* seems considerable as the latter excretes a brown pigment and produces only small amounts of calcium 5-ketogluconate whereas the former does not form a soluble pigment and the medium becomes incrusted with large crystals. Another example is afforded by *A. rancens*, *A. ascendens* and *A. paradoxus*, which appear to be very different because the first forms considerable amounts of acid when growing on glucose, the second does not dissolve $CaCO_3$ at all, and the third, which also does not form acid, is in addition catalase negative, and this in itself is an exceptional character for an aerobic bacterium. Further, the three organisms are strikingly different in appearance and one can easily understand that separate specific names should have been given to each. However, when a thorough enzymic analysis was made of these strains, the picture became considerably simpler and the differences were seen to be confined to one or at most two enzymes. For example, *G. suboxydans* contains a strong 5-ketogluconate reductase but only a weak glucono-oxidase, so that nearly all gluconate has to be converted into 5-ketogluconate. *G. melanogenus* on the other hand has a strong glucono- and 2-ketoglucono-oxidase, so that gluconate is oxidized to 2-ketogluconate and 2:5-diketogluconate, the latter substance being converted (perhaps spontaneously) into the brown pigment. The relationship between *A. rancens*, *A. ascendens* and *A. paradoxus* is still simpler. A thorough analysis of their enzymic constitution has revealed that whereas *A. rancens* contains the same enzymes for carbohydrate metabolism as *A. ascendens* it possesses in addition glucose oxidase, which is responsible for the gluconic acid formation. *A. paradoxus* has the same enzymes as *A. ascendens*, but it lacks catalase. The seemingly impressive differences between these three strains can thus be reduced to two enzymes. For lack of space we shall refrain from citing other examples. The 45 strains of our collection could be arranged, within the two genera of *Acetobacter* and *Gluconobacter*, in a decreasing order of enzymic equipment. Several strains were of course identical, but the differences between each strain and its two immediate neighbours in this sequence consists in one or at most a few enzymes. There are no abrupt changes.

Concerning the value of the 'species' concept in the acetic acid bacteria there are thus two alternative courses. Either the species concept can be retained, with the implication that a species name may imply a difference of only one or two enzymes, or the existing species names may be dropped and closely related 'species' regarded merely as varieties of a central biotype. We believe that the latter is the more logical attitude. We have therefore proposed (De Ley, 1961) that the acetic acid bacteria should be divided into two groups of morphologically nearly identical and physiologically closely related strains, to be called *Gluconobacter oxydans* and *Acetobacter aceti*. These are not type species but biotypes in the sense of Winogradsky (1952). The former includes polarly flagellate or nonflagellate strains with properties ranging from those of *G. liquefaciens* to those of *G. viscosus* and the latter includes the strains ranging from *A. xylinum* to *A. peroxydans*. The former species names are thereby reduced to the rank of catalogue numbers and indicate merely a set of enzymic properties. For the sake of clarity and for historical reasons the strains could be labelled as *A. aceti* (peroxydans), *A. aceti* (xylinum), *G. oxydans* (suboxydans), etc. It seems likely that the concept of biotypes approaches most closely the natural classification of bacteria.

It is not so easy for the moment to classify the new strains described by Wiame, Harpigny & Dothey (1959) under the name of *A. acidophilus*. These bacteria are apparently closely related to *A. paradoxus* and *A. peroxydans* because they are catalase negative and are unable to oxidize sugars. They can be regarded as the most specialized of all, since they oxidize only primary alcohols. They are unique in that they grow only in the pH range 2·7–4·0 and appear to require CO_2.

(d) *A proposal for a possible phylogeny*

The above mentioned finding, that within each biotype all the strains investigated can be arranged in a nearly continuous sequence of complexity of enzymic outfit, has enabled a suggestion to be made about their possible phylogenetic relationship, employing the modern biological concept of 'regressive physiological evolution' (De Ley, 1961). Briefly, one can imagine that an ancestor with the genes or enzymes controlling the properties . . . CDEF lost the property F through mutation. The new type . . . CDE again lost a property, E, giving rise to . . . CD, from which later the type . . . C originated, and so on. Each population could persist with its properties unchanged, so that at the present moment a series of types would exist with the properties . . . CDEF, . . . CDE, . . . CD, . . . C, etc. This is precisely the situation which exists in the acetic acid bacteria, as the example given in Table 2 shows. One can evidently imagine for example that *A. aceti* Ch 31 originated from an ancestor similar to *A. mesoxydans* 4 by a mutation involving a weakened activity of fructo- and gluconokinase, and that an ancestor similar to *A. mobilis* 6428 completely lost

Table 2. *The enzymes of some acetic acid bacteria, indicating a sequence of decreasing enzymic outfit*

Organism	Fructose and gluconate oxidation	Mannitol dehydrogenase	Gluconokinase	Hexokinase	Galactose and xylose oxidase	Glucose oxidase	Catalase	Lactate and ethanol oxidation
A. mesoxydans 4	+	+	+	+	+	+	+	+
A. aceti Ch 31	weak	+	+	+	+	+	+	+
A. mobilis 6428	—	weak	+	+	+	+	+	+
A. turbidans 6424	—	—	+	+	+	+	+	+
A. estunensis E	—	—	weak	+	+	+	+	+
A. lovaniensis 13	—	—	—	—	+	+	+	+
A. rancens 15	—	—	—	—	—	+	+	+
A. ascendens A	—	—	—	—	—	—	+	+
A. paradoxus 30	—	—	—	—	—	—	—	+

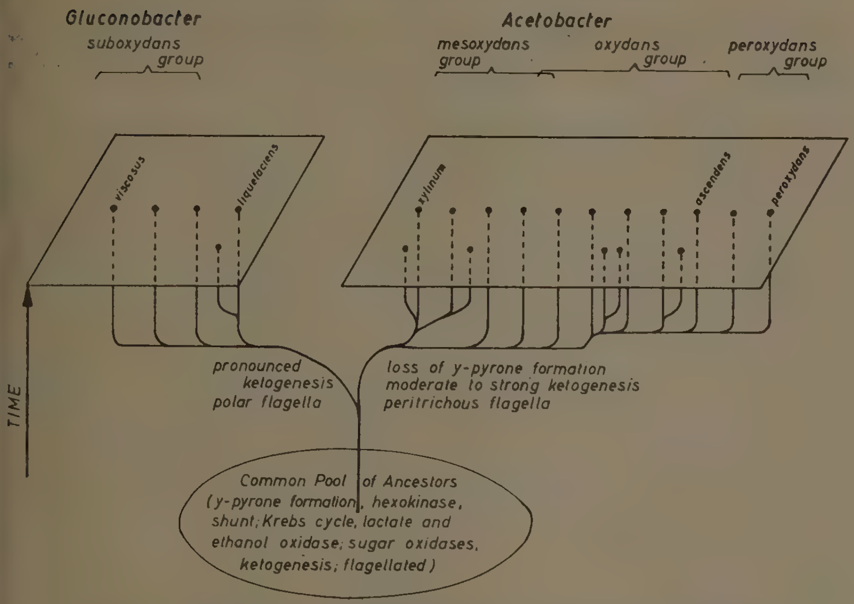

Gluconobacter
suboxydans group
Acetobacter
mesoxydans group
oxydans group
peroxydans group
viscosus
liquefaciens
xylinum
ascendens
peroxydans
TIME
pronounced ketogenesis polar flagella
loss of y-pyrone formation moderate to strong ketogenesis peritrichous flagella
Common Pool of Ancestors (y-pyrone formation, hexokinase, shunt; Krebs cycle, lactate and ethanol oxidase; sugar oxidases, ketogenesis; flagellated)

Fig. 5. A suggested phylogenetic relationship for some acetic acid bacteria: the possible origin of the bacteria from our collection, based mainly on comparative carbohydrate metabolism. For details see Figs. 6 and 7. (From De Ley, 1961).

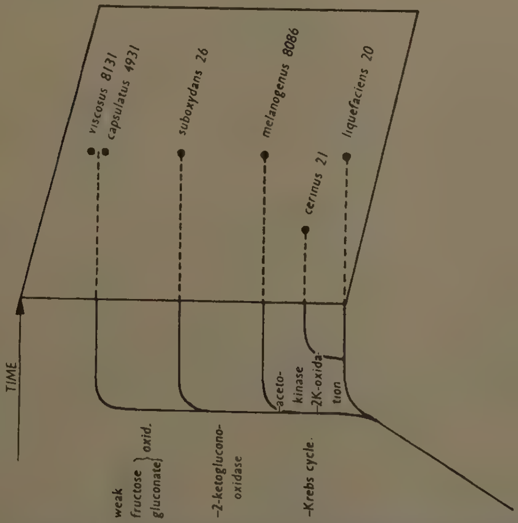

viscosus 8/31
capsulatus 4931
suboxydans 26
melanogenus 8086
cerinus 21
liquefaciens 20
TIME
-aceto-kinase
-2K-oxidation
weak fructose } oxid. gluconate}
-2-ketoglucono-oxidase
-Krebs cycle

Fig. 6. Detail of the scheme in Fig. 5. The *Gluconobacter* biotype. (From De Ley, 1961). A minus sign preceding a property indicates loss of that property.

the mannitol dehydrogenase and gave rise to *A. turbidans* 6424. A possible phylogenetic relationship among some of the strains which we studied is presented in Figs. 5, 6 and 7.

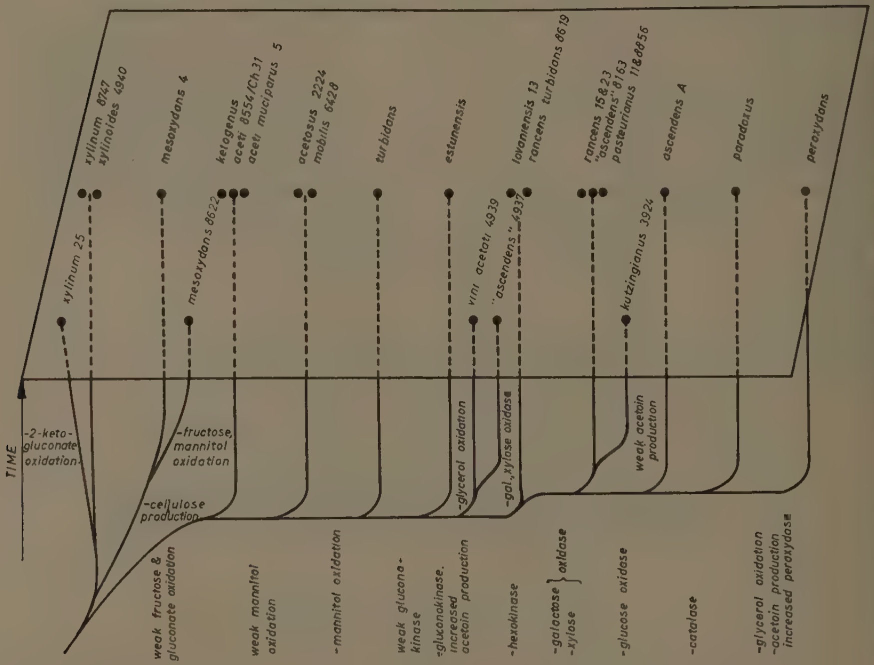

xylinum 8747
xylinoides 4940
mesoxydans 4
ketogenus
aceti 8554/Ch 31
aceti muciparus 5
acetosus 2224
mobilis 6428
turbidans
estunensis
lovaniensis 13
rancens turbidans 8619
rancens 15&23
"ascendens" 8163
pasteurianus 11&8856
ascendens A
paradoxus
peroxydans
xylinum 25
mesoxydans 8622
vini acetati 4939
"ascendens" 4937
kutzingianus 3924
TIME
-2-keto-gluconate oxidation
-fructose, mannitol oxidation
-cellulose production
-glycerol oxidation
-gal., xylose oxidase
weak acetoin production
weak fructose & gluconate oxidation
weak mannitol oxidation
-mannitol oxidation
weak glucono-kinase
-gluconokinase, increased acetoin production
-hexokinase
-galactose
-xylose
oxidase
-glucose oxidase
-catalase
-glycerol oxidation
-acetoin production
increased peroxydase

Fig. 7. Detail of the scheme in Fig. 5. The *Acetobacter* biotype. (From De Ley, 1961). A minus sign preceding a property indicates loss of that property.

The isolation of several mutants lends strength to this hypothesis. Most of the known mutants of acetic acid bacteria are loss mutants. In accordance with the principle of the phylogenetic hypothesis *A. xylinum* loses the ability to synthesize cellulose and is thus transformed to *A. mesoxydans* (Schramm & Hestrin, 1954; Shimwell & Carr, 1958). *A. pasteurianus* often loses its power to produce the starch-like polysaccharide and becomes indistinguishable from *A. rancens* (Shimwell, 1957). Shimwell (1959) isolated several mutants which nearly always represented losses, e.g. *A. mesoxydans* to *A. rancens* (loss of oxidation of glycerol to dihydroxyacetone), and *A. ascendens* to *A. paradoxus* (loss of catalase). In our laboratory we have extensively studied the enzymic differences between several mutants and their parents. We have observed the mutation of *G. melanogenus* into *G. suboxydans* by the loss of glucono-oxidase and 2-ketogluconoreductase (De Ley & Stouthamer, 1959), of *A. rancens* into *A. ascendens* through the loss of glucose oxidase, and of *A. ascendens* into *A. paradoxus* due to the loss of catalase (Schell, 1960).

4. The Comparative Carbohydrate Metabolism of *Pseudomonas*

(a) *The inner metabolism*

(i) *Glycolysis, shunt and Entner-Doudoroff pathway.* Several *Pseudomonas* species have been found to lack a complete Embden-Meyerhof system. A first indication was obtained through the studies of Campbell & Norris (1950), who analyzed the phosphorus fraction of cells of *Ps. aeruginosa* and found no evidence for the presence of glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and fructose-1:6-diphosphate. The enzymic aspects of glucose metabolism in *Ps. fluorescens* were studied extensively by Wood & Schwert (1953*a*,*b*, 1954) and Kovachevich & Wood (1955*a*,*b*). These authors pointed out that the Embden-Meyerhof mechanism could not function because of the lack of phosphofructokinase. On the other hand the complete system for the shunt and the Entner-Doudoroff pathway was present. It was found that several enzymes were particle bound. Since these particles also contained the complete cytochrome system we believe them to be oxidosomes, and thus to be part of the cytoplasmic membrane. On this basis the results of Wood *et al.* have been interpreted and a summary is given in Fig. 8. It is to be noticed that this strain of *Ps. fluorescens* metabolizes glucose by way of 2-ketogluconate, and the C_6-chain is introduced into the shunt by way of 2-ketogluconokinase and 2-ketogluconate-6-phosphate reductase. It could not happen directly by way of glucose or gluconate because the corresponding kinases are absent. The presence or absence of each of

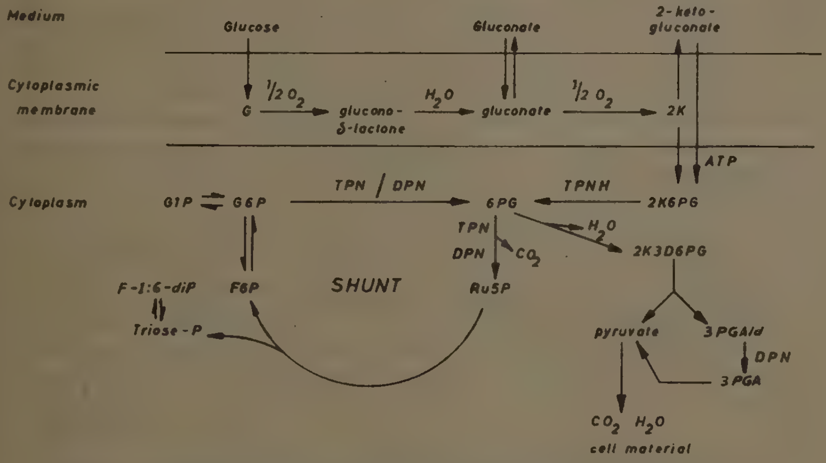

Medium
Glucose
Gluconate
2-keto-gluconate
Cytoplasmic membrane
G
½ O2
glucono-δ-lactone
H2O
gluconate
½ O2
2K
ATP
Cytoplasm
G1P
G6P
TPN / DPN
6PG
TPNH
2K6PG
TPN
DPN
CO2
H2O
2K3D6PG
F-1:6-diP
F6P
SHUNT
Ru5P
Triose-P
pyruvate
3PGAld
DPN
3PGA
CO2 H2O
cell material

Fig. 8. Carbohydrate metabolism in *Pseudomonas fluorescens*, according to the results of Wood *et al.* (see text), and a proposal for the localization of enzymes in this organism. Explanations of abbreviations are given in Appendix 3.

the three kinases is probably specific for each particular strain, because kinases for glucose, fructose and mannose have been demonstrated in *Ps. aeruginosa* (Eagon & Williams, 1960).

Hochster & Katznelson (1958) suggested that the Entner-Doudoroff pathway may be part of a cyclic mechanism in the phytopathogenic organism *Xanthomonas phaseoli*, and possibly in other bacteria which lack phosphofructokinase and have only a weak shunt. Their proposal is summarized in Fig. 9.

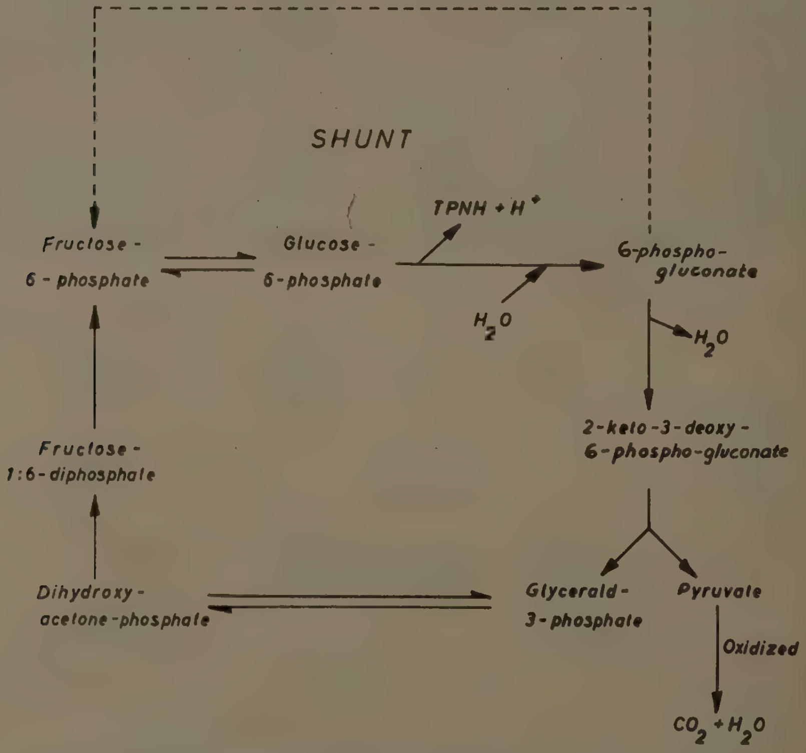

SHUNT
Fructose-6-phosphate
Glucose-6-phosphate
TPNH + H+
H2O
6-phospho-gluconate
H2O
2-keto-3-deoxy-6-phospho-gluconate
Fructose-1:6-diphosphate
Dihydroxy-acetone-phosphate
Glycerald-3-phosphate
Pyruvate
Oxidized
CO2 + H2O

Fig. 9. Intermediary carbohydrate metabolism in *Xanthomonas phaseoli*, according to Hochster & Katznelson (1958).

Two strains of *Pseudomonas*, used by Wang *et al.* (1958), oxidized 71% and 88% respectively of the glucose by way of the Entner-Doudoroff pathway, the rest by way of the shunt. Gluconate on the other hand was used exclusively by the Entner-Doudoroff pathway (Wang, Stern & Gilmour, 1959). The Entner-Doudoroff pathway plays a predominant role in the glucose catabolism of *Ps. aeruginosa*, *Ps. reptilivora* and *Zymomonas mobilis* (Stern, Wang & Gilmour, 1960). The enzymes of the latter pathway were also detected in several species of *Xanthomonas* (Katznelson, 1958), *Rhizobium* and *Azotobacter* (Katznelson, 1955). The strain of *Ps. fluorescens* used by Stodola *et al.* (see below) for the production of α-ketoglutarate was investigated by Lewis *et al.* (1955) using labelled glucose, and it appeared from their data that glycolysis occurred to a very small extent only, that the shunt was the major pathway, accounting for one half or more of the glucose molecules catabolized, and that the remaining substrate was dealt with according to the Entner-Doudoroff pathway.

(ii) *Krebs cycle, glyoxylate cycle.* The mechanism of acetate oxidation by *Ps. fluorescens* was elucidated mainly by Kornberg *et al.* The function of the Krebs cycle is not only to provide energy from the oxidation of acetate, but also to supply the carbon skeleton for amino acid synthesis. Acetate condenses with oxaloacetate, passes through the Krebs cycle and yields CO_2, H and the same amount of oxaloacetate. It was pointed out by Kornberg (1958) that "... any removal of intermediates from the cycle would prevent the regeneration of oxaloacetate and would therefore cause the cycle to stop". Thus a mechanism has to be present to replenish the intermediates of the cycle. *Ps. fluorescens* KB1 rapidly oxidizes acetate and all the intermediates of the Krebs cycle, which confirms that the latter cycle operates in this organism. When 2-C^{14}-acetate was used, it was shown that over 70% of the total isotope was initially incorporated in the C_4 compounds and primarily into malate without passing through citrate. Therefore, acetate enters the tricarboxylic acid cycle at two sites, to form citrate at one and malate at the other. Malate synthetase, which had been discovered in *E. coli*, effects the condensation

$$\text{glyoxylic acid} + \text{acetyl-CoA} + H_2O \longrightarrow \text{malic acid} + \text{CoA-SH}.$$

Glyoxylic acid, required for this reaction, is provided by the enzyme *iso*citratase. The first indication for the existence of this enzyme was discovered by Campbell, Smith & Eagles (1953). They found that dried cells of *Ps. aeruginosa* were able to oxidize nearly all the usual intermediates of the Krebs cycle. These cells contain the condensing enzyme. The oxidation of succinate, fumarate and malate proceeds by way of oxaloacetate. However, citrate is not converted to α-ketoglutarate. It was found that either *cis*aconitate or citrate yields glyoxylate and succinate and that *iso*citrate is not involved. Saz & Hillary (1956) indeed later found that the C_6 tricarboxylic acids undergo this split in *Ps. aeruginosa* and *Ps. fluorescens* and advanced the view that (+)-*iso*citrate is the real substrate. Later, studies by several authors disclosed that in fact an aldol cleavage of *iso*citrate is involved, catalyzed by the enzyme *iso*citratase:

$$\textit{iso}\text{citric acid} \longrightarrow \text{succinic acid} + \text{glyoxylic acid}.$$

Both reactions were readily demonstrated by Kornberg & Madsen (1958) in *Ps. fluorescens.* In conjunction with the known reactions, which lead from citrate to *iso*citrate and from malate to oxaloacetate, this new mechanism represents a variant of the Krebs cycle. The result is the net synthesis of one molecule of C_4 dicarboxylic acid from two molecules of acetate. This new cycle, which has been called the 'glyoxylate cycle', is represented in Fig. 10. The operation of the glyoxylate cycle avoids the loss of carbon as CO_2 which otherwise occurs through the actions of the oxalosuccinic decarboxylase and the oxidative α-ketoglutaric decarboxylase. For a complete review of our knowledge of this cycle see Kornberg (1959).

*Iso*citratase and malate synthetase are formed when the organisms are grown on acetate and are thus inducible enzymes. This was confirmed by Madsen & Hochster (1959) with *Xanthomonas phaseoli*. This bacterium, grown on glucose, contains all the individual enzymes of the Krebs cycle. However, when grown on acetate, both *iso*citratase and malate synthetase are also present. Most of the Krebs cycle enzymes were detected in *Ps. nigrifaciens* by Robinson & Katznelson (1956).

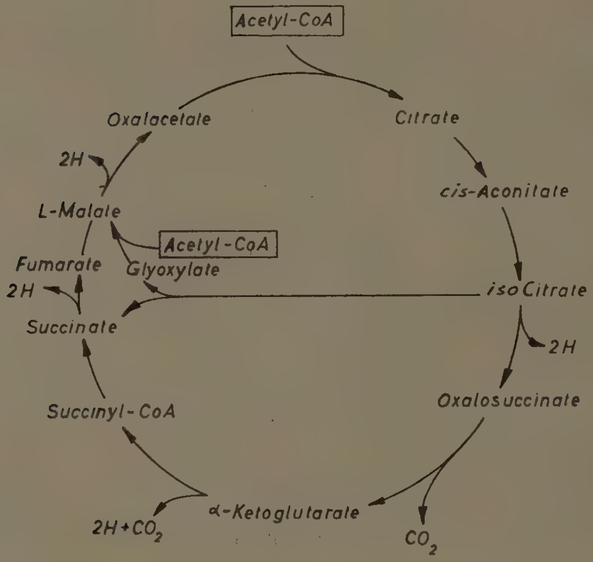


Fig. 10. The relationship between the Krebs cycle and the glyoxylate cycle.

The oxidation of the intermediates of both cycles is complicated by permeability phenomena. Kogut & Podoski (1953) and Barrett & Kallio (1953) showed that cell-free extracts of fluorescent pseudomonads oxidized several Krebs cycle intermediates rapidly and at once, whereas with intact organisms there was a lag period before a linear oxidation rate was reached. Clarke & Meadow (1959) confirmed and extended these observations. In addition, they secured evidence to support the hypothesis that the first step in the uptake of pyruvate, malate, α-ketoglutarate, citrate, acetate, fumarate and succinate consists in the action of inducible permeases. The latter enzymes are no exceptions in *Pseudomonas*, any more than in other bacteria. Shilo & Stanier (1957) found that the metabolism of the tartaric acids by some fluorescent pseudomonads requires specific and inducible transport systems for bringing these substrates into the cell.

(iii) *Unusual catabolic reactions*. Some *Pseudomonas* spp. carry out unusual catabolic reactions linked with the Krebs cycle. Only the oxidation of malonate, and of oxalate and formate, will be considered.

The oxidation of malonate. Malonate is a well known inhibitor of succinic dehydrogenase and therefore a much-used inhibitor of the Krebs cycle. However, this substance is of little use for the evaluation of the latter mechanism in pseudomonads, since several strains can oxidize it nearly to completion. Decarboxylation precedes oxidation, ATP, CoA and Mg^{++} being required for the decarboxylation (Hayaishi, 1953; Wolfe, Ivler & Rittenberg, 1954). Although the mechanism is not yet entirely clear, several important contributions to its understanding have been made by Yamada & Jacoby (1959, 1960). Malonic semialdehyde has been established as the product of hydration of acetylene monocarboxylic acid in a reaction catalyzed by enzymes of *Ps. fluorescens*. Subsequently, an enzyme was isolated, malonic semialdehyde oxidative decarboxylase, which mediates the reaction

$$\text{malonic semialdehyde}+\text{DPN}^{+}+\text{CoA} \longrightarrow \text{acetyl-CoA}+\text{CO}_2+\text{DPNH}+\text{H}^{+}.$$

In this way malonate is ultimately oxidized by way of the Krebs cycle.

The oxidation of oxalate and formate. Ps. oxalaticus is able to grow on either formate or oxalate as the sole source of carbon and energy. Both substrates are highly oxidized and it seemed not improbable that they are first oxidized to CO_2 which is subsequently fixed by a mechanism similar to those operating in photosynthetic and autotrophic organisms. This is so for formate but not for oxalate. Quayle & Keech (1956*a*,*b*) showed that formate is first oxidized by particle-linked enzymes (oxidosomes ?) and $C^{14}O_2$ is fixed primarily into phosphoglyceric and malic acids. In cell-free extracts phosphoribulokinase and carboxydismutase were detected: these were responsible for the reactions

$$\text{ribulose-5-phosphate} \xrightarrow{\text{ATP}} \text{ribulose-1:5-diphosphate} \xrightarrow{\text{CO}_2} \text{2:3-phosphoglyceric acid.}$$

Carbon dioxide can also be fixed into pyruvate or phosphopyruvate according to the reactions

$$\text{pyruvate}+\text{CO}_2+\text{TPNH}+\text{H}^{+} \longrightarrow \text{malate}+\text{TPN}^{+}$$
$$\text{phosphopyruvate}+\text{CO}_2+\text{IDP} \longrightarrow \text{oxaloacetate}+\text{ITP},$$

where IDP and ITP stand for inosine di- and triphosphate respectively. Cells grown on oxalate apparently oxidize this substrate along different lines (Kornberg, 1959). This view is strengthened by the observations of Jayasuriya (1956) that citrate accumulates when the oxidation of glycollate is blocked by fluoroacetate. The same happens with glycine and several Krebs cycle intermediates, but not with acetate or oxalate. Glycollate appears to act as the sole source of carbon for citrate, the rest being oxidized to CO_2. The C_2 of glycollate is preferentially incorporated. Quayle & Keech (1959*c*) found that carboxydismutase was absent when *Ps. oxalaticus* was grown on oxalate. The Calvin cycle can thus not function for the conversion of oxalate into cellular material. These results were recently confirmed and extended (Quayle & Keech, 1960) with C^{14}-oxalate, C^{14}-formate and $NaHC^{14}O_3$. C^{14}-oxalate was incorporated in 3 seconds into glycine and 3-phosphoglycerate. Cell-free extracts contain the enzyme for the reductive condensation of glyoxylate with the formation of glyceric acid and CO_2 according to the reaction

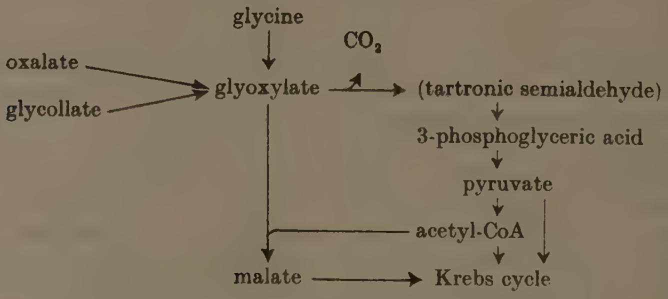
$$2\ \text{glyoxylate}+\text{DPNH} \longrightarrow \text{glycerate}+\text{CO}_2+\text{DPN}.$$

These reactions are similar to those found in glycollate-grown *Ps. ovalis* by Kornberg & Gotto (1959) and postulated in a pseudomonad grown on glycine (Callely & Dagley, 1959). It seems, moreover, not unlikely that glyoxylate is formed in the following fashion:

$$\text{oxalate}+\text{acetyl-CoA} \longrightarrow \text{oxalyl-CoA}+\text{acetate}$$

$$\text{oxalyl-CoA}+\text{DPNH} \longrightarrow \text{glyoxylate}+\text{CoA}+\text{DPN}.$$

The metabolism of these C_2 compounds may thus be tentatively summarized as follows (Kornberg, 1959):

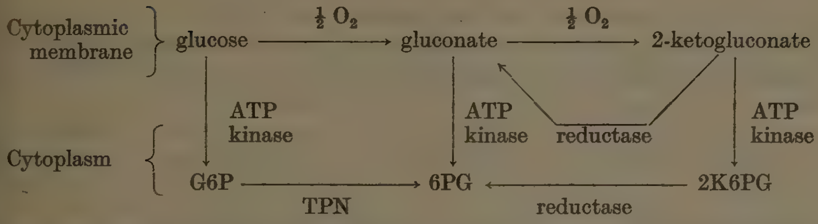


(b) *The outer metabolism: the oxidative properties*

From the point of view of carbohydrate metabolism, the genus *Pseudomonas* is characterized by the formation of five main end products in aerobic fermentations: gluconate from glucose; 2-ketogluconate from gluconate; pentonic acids from pentoses; malto- and lactobionic acids from the corresponding disaccharides, and α-ketoglutaric acid. The oxidation of polyols might be another characteristic. We shall discuss each briefly and separately.

(i) *The production of gluconate from glucose.* Alsberg (1911) observed the production of gluconic acid from a nutrient glucose solution by *Ps. savastanoi.* Later Pervozvanskii (1939) reported that fluorescent pseudomonads produced gluconate and 2-ketogluconate. With *Ps. putida* gluconic acid is the main end product (Pervozvanskii & Ivashkevich, 1939). Lockwood, Tabenkin & Ward (1941) found that most strains of *Pseudomonas* oxidize gluconic acid further into 2-ketogluconic acid. Only *Ps. ovalis* forms gluconic acid in considerable yield, namely 92%. This is practically identical to the yield of gluconic acid from glucose by *Acetobacter rancens* (Hermann & Neuschul, 1931). Four species of *Phytomonas* out of eight studied produced gluconic acid in yields varying from 40–82%. None of the latter strains produced 2-ketogluconate.

Entner & Stanier (1951) investigated some details of the oxidation of glucose by *Ps fluorescens.* In view of our present knowledge their results can now be more clearly understood. It is very likely that these cells possess a dual mechanism for glucose catabolism, as these authors suspected. Cells grown on asparagine oxidize glucose at once, at least partially to gluconate, and inducible enzymes participate in the oxidation of gluconate and 2-ketogluconate. Cells grown on glucose oxidize glucose and gluconate quickly and 2-ketogluconate without a noticeable induction period. It is very probable that in this species the oxidations occur in part on the cyotplasmic membrane, and the system can be represented as follows:



where ATP stands for adenosine triphosphate, G6P for glucose-6-phosphate, 6PG for 6-phosphogluconate, 2K6PG for 2-keto-6-phosphogluconate and TPN for triphosphopyridine nucleotide.

One can calculate from Fig. 2 of the paper by Entner & Stanier (1951) that the reactions on the cytoplasmic membrane capture about 75% of the glucose and 82% of the gluconate, with the formation of 2-ketogluconate in each case. The remainder is metabolized by the shunt mechanism to CO_2 and H_2O. From that moment on only 2-ketogluconate remains and is oxidized.

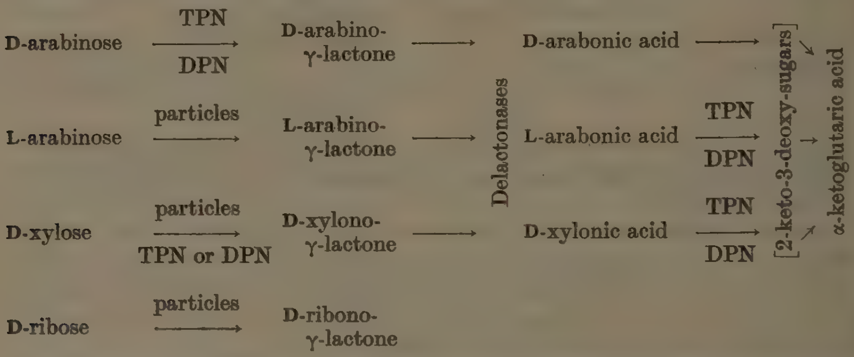
Ps. aeruginosa also forms gluconate and 2-ketogluconate from glucose (Norris & Campbell, 1949). By drying the cells *in vacuo* over P_2O_5 the dissimilative pathway to 2-ketogluconate is retained (Stokes & Campbell, 1951). It seems likely that the particulate glucose oxidase is also responsible for the oxidation of 2-deoxyglucose to 2-deoxygluconic acid by this organism (Williams & Eagon, 1959).

(ii) *The production of 2-ketogluconate from gluconate or glucose.* As mentioned above, Lockwood, Tabenkin & Ward (1941) found that, instead of gluconic acid, mainly 2-ketogluconic acid accumulated. The *Phytomonas* strains studied did not produce the latter compound. *Ps. fluorescens* converted 82% of the glucose in a 10% solution into 2-ketogluconic acid, and Wood & Schwert (1953*b*) found the 2-ketogluconate producing enzymes to be localized on submicroscopical particles, which we consider as parts of the cytoplasmic membrane. These particles (oxidosomes) were obtained from ultrasonically disrupted cells. Eagon (pers. comm.) crushed this strain with alumina, whereby much more of the glucose and gluconate oxidases appeared in the Spinco-supernatant: this could possibly be ascribed to breakage of the particles.

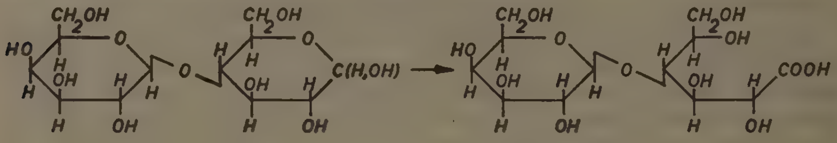
Ps. chromospirans (a species apparently rather closely related to *Ps. aeruginosa*) is able to oxidize a mixture of calcium D-gluconate and calcium L-idonate to the corresponding 2-keto acids. 2-Ketogluconate is metabolized further (presumably by way of the reductases) and calcium 2-keto-L-idonate is left untouched. It may be used for the synthesis of vitamin C (Färber, Vondrova, Liebster & Lubsik, 1958).

The report that two *Pseudomonas* strains seemed to be able to convert glucose into 5-ketogluconate (Stewart, 1959) requires further investigation.

(iii) *The production of pentonic acids from pentoses.* Several authors have reported that acids are produced from pentoses by members of the genus *Pseudomonas* (Chernomordik, 1939; Sears & Gourley, 1928). Lockwood & Nelson (1946) found that many pseudomonads can oxidize pentoses, such as D- and L-arabinose, D-xylose and D-ribose, to the corresponding pentonic acids. Bentley & Slechta (1960) found that pentoses are oxidized by particle-linked enzymes (oxidosomes ?). However, this is not a general rule. Weimberg (1960) reported that *Ps. fragi* is able to grow on D-ribose, D-xylose, and D- and L-arabinose, specific inducible enzymes being involved. The oxidation reactions can be represented as follows:


D-arabinose
TPN
DPN
D-arabino-γ-lactone
D-arabonic acid
L-arabinose
particles
L-arabino-γ-lactone
L-arabonic acid
TPN
DPN
D-xylose
particles
TPN or DPN
D-xylono-γ-lactone
D-xylonic acid
TPN
DPN
D-ribose
particles
D-ribono-γ-lactone
Delactonases
[2-keto-3-deoxy-sugars]
α-ketoglutaric acid

(iv) *The production of bionic acids from the corresponding disaccharides, and their breakdown.* Several *Pseudomonas* species carry out an unusual type of disaccharide breakdown, in which these substances are oxidized to the corresponding bionic acids. For example, lactose is converted to lactobionic acid.



Lactose Lactobionic acid

Stodola & Lockwood (1947) provided the first clear cut example of a biochemical oxidation of reducing disaccharides without prior hydrolysis. They reported that 10 out of 15 species produced acid from lactose. *Ps. graveolens* proved to be by far the best, and was able to oxidize maltose to maltobionic acid with a yield of 77% in 50 hr.

These results were extended by Kluyver, De Ley & Rijven (1951), who found that 4 out of 19 species were excellent producers of bionic acids. *Ps. quercito-pyrogallica* oxidizes lactose and maltose, *Ps. calco-acetica* and *Ps. fluorescens* oxidize lactose and *Ps. aromatica* oxidizes maltose. In most of the cases 90—95% yields of bionic acids were obtained in 2–4 days on a shaking machine. Masuo, Shima & Yoshida (1952) reported 50—60% yields of calcium lactobionate from lactose by the action of *Ps. gluconicum* and *Ps. woodsii*. Using the same strains as Kluyver, De Ley & Rijven (1951), Bentley & Slechta (1960) fully confirmed the results of the previous authors. In addition cellobiose and melibiose were found to be oxidized to the corresponding bionic acids. As could be expected from the general nature of the oxidative reactions, Bentley & Slechta found disaccharides and several monosaccharides to be oxidized by particle-bound enzymes (oxidosomes ?). The electrons are transferred to the cytochrome system, which is located on the same structure. Flavins do not seem to be involved, since no H_2O_2 was detected. All this is closely similar to the oxidation of monosaccharides and their derivatives which we studied with the oxidosomes of acetic acid bacteria. This similarity extends further than the mere enzymic constitution. Acetic acid bacteria often do not derive any profit from growth in the presence of substrates which are oxidized by processes which occur only on the cytoplasmic membrane: often they cannot use such substrates as carbon sources and are apparently unable to couple the energy derived from the oxidation. For example, nearly all acetic acid bacteria convert galactose and xylose quantitatively to the corresponding acids, but they grow at the expense of the other constituents of the nutritive medium. The same holds for the oxidation of glucose to gluconic acid by *A. rancens* (De Ley, 1960, 1961). Kluyver, De Ley & Rijven (1951) found that there was nearly quantitative conversion of maltose and lactose into the corresponding bionic acids and Bentley & Slechta (1960) observed that growth in the presence of the disaccharides did not differ significantly from that observed with peptone alone. We therefore postulate here that the particulate enzymes for the oxidation of mono- and disaccharides to the -onic acids are located on the cytoplasmic membrane.

The formation of bionic acids has also been observed with other micro-organisms: *Bacterium anitratum* (Villecourt & Blachère, 1955), *Penicillium chrysogenum* (Cort *et al.* 1956) and the red alga *Iridophycus flaccidum* (Bean & Hassid, 1956).

Most of the strains studied by Kluyver, De Ley & Rijven (1951) brought about a slow disappearance of the bionic acids as the cultures aged. This was taken as an indication of the splitting of the oxygen bridge in the molecule by a hydrolase. This is indeed very likely because Stodola & Lockwood (1947) found that the greater part of their strains were capable of hydrolyzing maltose. A clear cut case of the hydrolysis of lactobionate by β-galactosidase was studied by Bernaerts & De Ley (1957) with *Corynebacterium simplex*. The earlier work on bionate hydrolysis was reviewed by De Ley & Bernaerts (1952) and microbiological decomposition of bionates was studied extensively by Bernaerts & De Ley (1952) and Bernaerts (1953): but this is beyond the scope of the present discussion. The production of 3-ketoglycosides from bionates will be discussed below.

(v) *The production of α-ketoglutaric acid.* Lockwood & Stodola (1946) discovered this compound among the end products of glucose oxidation by *Ps. fluorescens* B-6. About one-fifth of the glucose oxidized was recovered as the acid. Koepsell (1950) studied the details of gluconate oxidation by this bacterium and found that in the absence of iron it was nearly quantitatively converted to 2-ketogluconate, which later disappeared slowly with the formation of pyruvate, α-ketoglutarate and CO_2. The oxidation was dependent on trace elements. In the presence of iron, gluconate was completely oxidized. Keto acids were only formed when net cell production had ceased, and thus appeared to result from the activity of resting cells, so that they could be regarded as an 'overflow' system or a disconnected biosynthetic pathway. A typical fermentation balance can be recalculated from Koepsell's results and expressed as the reaction

$$\begin{aligned}1\ \text{gluconate} + 2{\cdot}38\ O_2 \longrightarrow\ & 0{\cdot}1\ \text{2-ketogluconate} + 0{\cdot}36\ \alpha\text{-ketoglutarate}\\ & + 0{\cdot}42\ \text{pyruvate} + 2{\cdot}22\ CO_2 + 3{\cdot}57\ H_2O.\end{aligned}$$

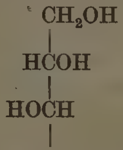
This shows that 1 mole of gluconate gives rise to approximately 0·4 mole of α-ketoglutarate, 0·5 mole of pyruvate and 2·5 moles of CO_2. In further studies higher yields were obtained, ranging from 0·5–0·55 mole of α-ketoglutarate/mole of glucose (Koepsell, Stodola & Sharpe, 1952). It seems not impossible that still higher yields could be obtained. One can imagine that glucose is metabolized by two pathways, in one of which it is rapidly converted into gluconate and 2-ketogluconate, possibly by enzymes of the cytoplasmic membrane, and in the other more slowly by way of the kinases into the shunt or a similar mechanism. After all the glucose has disappeared, most of it has accumulated as 2-ketogluconate which can then be fed back into the shunt either by the system 2-ketogluconokinase and 2-keto-6-phospogluconate reductase or by the system 2-ketogluconoreductase and gluconokinase.

Koepsell, Stodola & Sharpe (1952) considered it unlikely that α-ketoglutarate is formed directly from the C_6 chain of the sugar molecule for two reasons: first, because pyruvate seems to give rise to α-ketoglutarate, and second, the sum of the pyruvate and the α-ketoglutarate cannot exceed 1, unless in addition some CO_2 were fixed into pyruvate. α-Ketoglutarate is thus apparently formed by way of the Krebs cycle. This was further confirmed by Kogut & Podoski (1953), who showed that *Ps. fluorescens* oxidized succinate incompletely and with the accumulation of α-ketoglutarate and pyruvate. The yield of the former end product was variable but could account for up to 70% of the oxygen consumed. α-Ketoglutarate was isolated from a growth medium with succinate as the sole carbon source. Most of the Krebs cycle enzymes were detected in this strain.

There appears to be an essential difference between the mechanism of α-ketoglutarate formation by *Ps. fluorescens* and by *Ps. saccharophila*. In the latter α-ketoglutarate is not formed by way of the Krebs cycle and incorporation of CO_2 into pyruvate, but is the end product of L-arabinose oxidation as previously explained (see Section 2b).

(vi) *The oxidation of sugar alcohols.* The oxidation of polyols has been particularly well studied with growing cultures of acetic acid bacteria. Its elucidation was the basis of Bertrand's rule. It is therefore rather surprising that so little work has been carried out along similar lines with the closely related *Pseudomonas*. Shaw (1956) studied the oxidation of a number of polyols by an organism tentatively identified as a fluorescent *Pseudomonas* and found them to be oxidized by inducible DPN-linked dehydrogenases with the formation of ketoses. This author accepted the existence of two enzymes, galactitol dehydrogenase and D-iditol dehydrogenase.

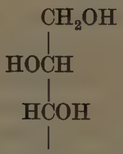
The former accounts for the oxidation of polyols with an L-threo configuration



$$\begin{array}{c} CH_2OH \\ | \\ HCOH \\ | \\ HOCH \\ | \end{array}$$

It is induced by sorbitol and dulcitol and catalyzes the oxidation of dulcitol to D-tagatose, sorbitol to D-fructose, L-iditol to L-sorbose and L-arabitol to L-ribulose.

The D-iditol dehydrogenase accounts for the oxidation of polyols with a D-threo configuration



$$\begin{array}{c} CH_2OH \\ | \\ HOCH \\ | \\ HCOH \\ | \end{array}$$

It is induced by dulcitol and catalyzes the conversion of dulcitol to D-tagatose, sorbitol to fructose, D-gulitol to D-fructose, D-talitol to D-allulose and xylitol to L-xylulose.

(c) *Oxidosomes and the enzymic anatomy of the* Pseudomonas *cell*

Ultramicroscopic particles which are able to oxidize several substrates with the uptake of O_2 have been prepared from *Pseudomonas* (as well as from many other bacteria; see Alexander, 1956). The particles contain the complete cytochrome system. In accordance with the results obtained with the acetic acid bacteria and described above, we propose to give the name of 'oxidosomes' to these particles. It seems very probable that these oxidosomes represent either the disrupted outer solid envelope of the *Pseudomonas* cell or, and even more likely, the disrupted cytoplasmic membrane itself. We have mentioned above that in different *Pseudomonas* species the following particulate enzymes have been detected: glucose oxidase, gluconate oxidase, pentose oxidases, disaccharide oxidases and delactonases. Cytochromes b and c and cytochrome oxidases have been detected in the oxidosomes of several fluorescent pseudomonads (see Table 3). There exist now two convincing proofs that *Pseudomonas* oxidosomes are derived from the outer, solid cell envelope.

Table 3. *The cytochromes present in several strains of* Ps. fluorescens

Material examined	Absorption maxima (mμ) of cytochromes					Reference
	γ-peak	β-peak	α-peak			
			c	b_1	a_1	
Whole cells	427–430	527	555	560–562		Kogut (1957)
Whole cells	424	523	552	560	580	Smith (1954)
Oxidosomes	422	520	552	560–562		Stanier *et al.* (1953)
Oxidosomes	425	520–530	558	565		Wood & Schwerdt (1953*d*)
Deoxycholate-dissolved oxidosomes	425–428	524 (530; 550)	553	560		Hunt (1958)

The first concerns the oxidation of L-mandelic acid. Gunsalus, Gunsalus & Stanier (1953), Gunsalus, Stanier & Gunsalus (1953) and Stanier, Gunsalus & Gunsalus (1953) showed that L-mandelic acid is oxidized to benzoylformic acid by oxidosome-bound enzymes. These oxidosomes contain the complete cytochrome system. Nakada & Nozu (1958) showed definitely that these enzymes are localized on the 'ghost' of the protoplast. The second concerns the oxidation of nicotinic acid. Hunt (1958) showed that the oxidosomes from *Ps. fluorescens* KB 1 oxidize nicotinic acid to 6-hydroxynicotinic acid. Hunt, Rodgers & Hughes (1959) reinvestigated this system and found the bulk of its activity to be located on a structure resembling the cell wall and protoplast membrane of the cell.

Several other enzymes have been found to be associated with this particle or membrane fraction. According to Burrous & Wood (1959) the membrane fraction of *Ps. fluorescens* contains DPNH-oxidase, 2-keto-3-deoxy-6-phosphogluconate aldolase and 50% of the gluconic dehydrogenase activity. A particulate fraction from *Ps. oxalaticus* oxidized formate to CO_2 (Quayle & Keech, 1959*b*). According to Bernheim (1950) the sarcosine oxidase of *Ps. aeruginosa* (the enzyme which oxidizes sarcosine to glycine and formaldehyde) is an inducible enzyme, present on the outer envelope of the cell.

It appears that little or no energy is derived by these bacteria from the incomplete oxidations which are carried out on the cytoplasmic membrane. Campbell, Ramakrishnan, Linnes & Eagles (1956) showed that during the oxidation of glucose to gluconic acid and 2-ketogluconic acid by pseudomonads no high energy phosphate was formed and that the yield of cells on equimolar amounts of glucose, gluconate or 2-ketogluconate was identical. This made it clear that these bacteria do not gain energy from these oxidative steps, and this also appears to be true of many acetic acid bacteria when growing on carbohydrates such as galactose, mannose, pentoses and glucose (the latter for *A. rancens* only), as discussed above. One is thus forced to the paradox that, although high energy phosphates are produced mainly through the cytochrome system, the oxidations, which occur on the cytoplasmic membrane, are useless to the bacteria. They appear to be carried out because the enzymes happen to be present, and it may be that these enzymes represent an atavism. On the other hand they may indicate an attempt by these organisms to develop a new pathway for energy utilization, unrelated to glycolysis, the hexose monophosphate oxidative cycle or the Entner-Doudoroff mechanism.

(d) *The possibility of a 'type' metabolic map for* Pseudomonas

The genus *Pseudomonas* is identified by a set of characteristic morphological properties as described in the different contributions to this Symposium. May one expect that a thorough biochemical and enzymic analysis of many strains will show that a rigid 'type' metabolic map exists either for the genus as a whole or for well delineated species within the genus? This prospect seems to be rather improbable. The experience with the Enterobacteriaceae (Kaufmann, 1954; Edwards & Ewing, 1955) and the acetic acid bacteria (see above) in conjunction with the concept of the biotype according to Winogradsky (1952) allows it to be predicted that the different strains of *Pseudomonas* will have closely related metabolic maps, with several individual differences. One can expect them to form a cluster or constellation as variations around a central theme. A summary of the biochemical pathways for carbohydrate metabolism in the pseudomonads (mostly fluorescent ones) shows that the following mechanisms and features have been detected. (1) Glycolysis, which is at most of very low activity. (2) The hexose monophosphate oxidative cycle (shunt) and the Entner-Doudoroff pathway, which are the most important mechanisms for the catabolism of hexoses. (3) The Krebs cycle. (4) The glyoxylate cycle, the enzymes for which are inducible. (5) Terminal oxidation, which proceeds by way of the cytochromes. (6) The oxidation of glucose to gluconic acid. (7) The oxidation of gluconate to 2-ketogluconate. (8) Kinases for glucose, gluconate and 2-ketogluconate. (9) The oxidation of pentoses to the corresponding pentonic acids. (10) The oxidation of disaccharides to bionic acids. (11) The production of α-ketoglutaric acid from glucose. (12) The localization of several oxidases on the cytoplasmic membrane.

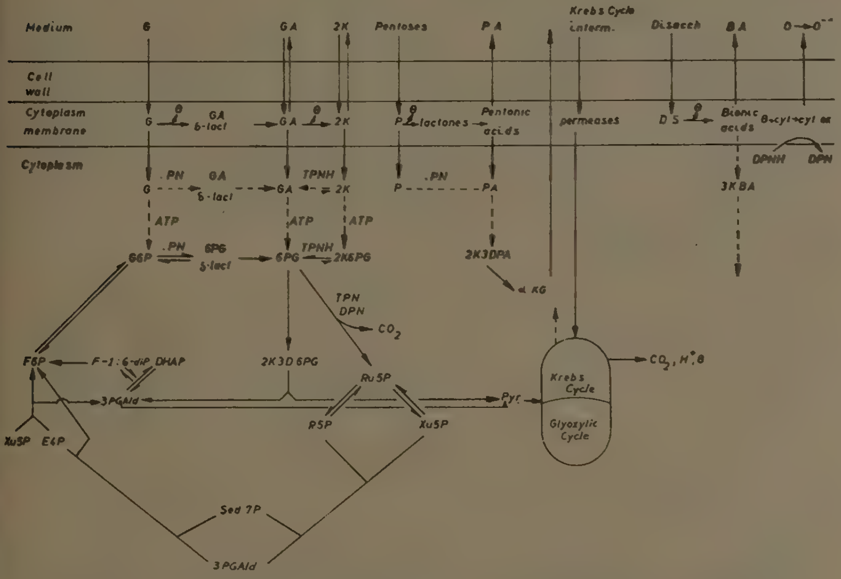

Medium
G
GA
2K
Pentoses
PA
Krebs Cycle interm.
Disacch
BA
O→O⁻⁻
Cell wall
Cytoplasm membrane
G
θ
GA δ-lacl
GA
θ
2K
P
θ
lactones
Pentonic acids
permeases
DS
θ
Bionic acids
θ-cyl→cyl ox
DPNH
DPN
Cytoplasm
.PN
GA
G
δ-lacl
GA
TPNH
2K
P
.PN
PA
3KBA
ATP
ATP
ATP
66P
.PN
6PG δ-lacl
6PG
TPNH
2K6PG
2K3DPA
α KG
TPN
DPN
CO2
F6P
F-1:6-diP
DHAP
2K3D 6PG
Ru5P
CO2, H2O
Krebs Cycle
Glyoxylic Cycle
3PGAld
Pyr
R5P
Xu5P
Xu5P
E4P
Sed 7P
3PGAld

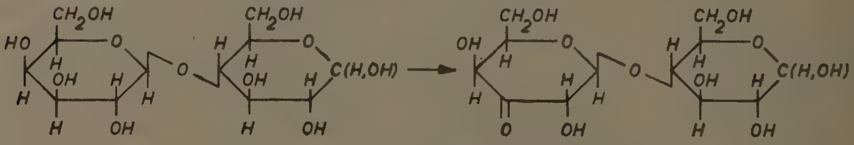
Fig. 11. A metabolic map of the reactions of the carbohydrate metabolism encountered in several pseudomonads and a proposal for the enzymic anatomy of these organisms. Explanations of abbreviations are given in Appendix 3.

Has one to expect that all these properties together constitute the criteria by which a strain can be allocated to the genus *Pseudomonas*? By no means. Though the properties (2)–(5) will probably be present in all the strains they are not typical, because these enzyme systems are widespread in many organisms. The virtual absence of glycolysis in *Pseudomonas* is unusual, because this mechanism is active in most other tissues. Again, the properties (6)–(12) are arbitrarily distributed over the different strains investigated, except for the glucose oxidase which seems to be present in most, if not all of the strains. In Fig. 11 the carbohydrate metabolism in *Pseudomonas* is summarized and a proposed enzymic anatomy is depicted.

5. Appendix 1. The Oxidation of Disaccharides and Bionates to 3-Ketoglycosides by Strictly Aerobic, Peritrichously Flagellated Bacteria

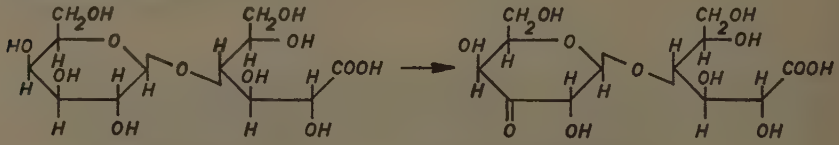
The oxidation of hexonic acids by strictly aerobic organisms usually proceeds at either C_2 or C_5. Pentonates are reported to be oxidized at C_2 only. The oxidation at carbons 3 and 4 has not been reported previously. A new type of sugar breakdown has been found, in which the first step consists in the oxidation of carbon 3 of disaccharides and bionates (Bernaerts & De Ley, 1958, 1960*a*,*b*). Several years ago we noticed that a strain isolated from river water was able to grow very well on bionates and produced a substance with reducing properties as strong as those of vitamin C (Bernaerts, 1953). The taxonomic position of this strain is not yet clear. We tentatively classified it as a saccharophilic *Alcaligenes faecalis*. The microbiological preparation of the new compounds was described, starting with lactose, maltose, and lacto- and maltobionate (Bernaerts & De Ley, 1960*a*), yields up to 75% being obtained. The compounds were formed very quickly, in 1–2 days, and disappeared as fast. They were obtained in a chromatographically pure state and their chemical constitution determined (Bernaerts & De Ley, 1958, 1960*b*). They turned out to be a new class of oxidation products, never detected before in metabolism and chemically unknown.

The oxidation of lactose may be represented as:



Lactose 4-O-β-3-keto-D-galactosido-D-glucose

and that of lactobionate as:



Lactobionic acid 4-O-β-3-keto-D-galactosido-D-gluconic acid

Similar compounds were obtained from maltose, maltobionate and several other disaccharides. The biochemical formation of these new compounds is interesting for three reasons. First, they point to a new and unsuspected pathway of disaccharide catabolism in bacteria. Second, the oxidation does not occur in the aglycone part, where one would expect it to happen (2- or 5-ketobionates), but in the glycosyl radical; and third, they constitute the first examples of 3-ketosugar derivatives isolated as intermediates in a metabolic pathway. It may be recalled that 3-keto-6-phosphogluconate has been postulated as an intermediate in the activity of 6-phosphogluconate dehydrogenase but has never been detected or isolated.

6. APPENDIX 2. CARBOHYDRATE METABOLISM OF THE AUTOTROPHIC *RHODOPSEUDOMONAS SPHEROIDES*

Szymona & Doudoroff (1960) have recently studied the carbohydrate metabolism of this micro-organism. We have summarized their results in Fig. 12, in which is also included a proposal for the enzymic anatomy of these cells. These authors have indeed isolated particulate enzymes, which oxidize a variety of sugars with the uptake of O_2. It is very probable that this fraction constitutes the oxidosomes.

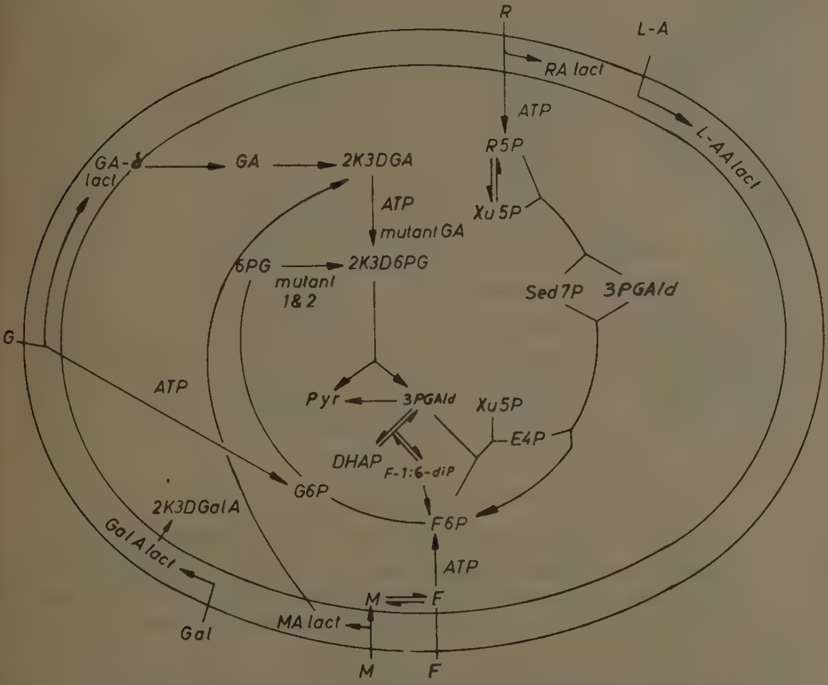

R
L-A
RA lact
L-AA lact
ATP
R5P
Xu 5P
GA-δ lact
GA
2K3DGA
ATP
mutant GA
6PG
2K3D6PG
mutant 1& 2
Sed 7P
3PGAld
G
ATP
Pyr
3PGAld
Xu5P
E4P
DHAP
F-1:6-diP
G6P
F6P
2K3DGalA
GalA lact
ATP
M
F
MA lact
Gal
M
F

Fig. 12. A metabolic map of the carbohydrate metabolism in *Rhodopseudomonas spheroides* (from the results of Szymona & Doudoroff, 1960), and a proposal for the enzymic anatomy of these organisms. Explanations of abbreviations are given in Appendix 3.

7. APPENDIX 3. LIST OF ABBREVIATIONS USED IN THE FIGURES

acetaldehyde	acetald
acetic acid	HAc
acetyl-coenzyme A	Ac-CoA
cis-aconitate	*cis*-acon
adenosine triphosphate	ATP
amyl alcohol	amyl
L-arabinose	L-A
L-arabonic acid lactone	L-AA lact
bionic acids	BA
n-butyric acid	n-but
citrate	Citr
cyanide	CN
cytochrome	cyt
cytochrome oxidase	cyt ox
dihydroxyacetone	DHA
dihydroxyacetone phosphate	DHAP
2:5-diketogluconate	2:5 dik
diphosphopyridine nucleotide	DPN
disaccharides	DS

APPENDIX 3—*continued*

electron	θ
erythrose-4-phosphate	E4P
fructose	F
fructose-1 : 6-diphosphate	F-1 : 6-diP
fructose-6-phosphate	F6P
fumarate	Fum
galactonic acid lactone	GalA lact
galactose	Gal
gluconate	GA
glucono-δ-lactone	GA δ-lact
glucono-δ-lactone-6-phosphate	6PG-δ-lact
glucose	G
glucose-1-phosphate	G1P
glucose-6-phosphate	G6P
glycerol	Glyc
α-glycerophosphate	α-GlycP
*iso*butyric acid	*iso*but
*iso*citrate	*iso*citr
3-ketobionates	3KBA
2-keto-3-deoxygalactonic acid	2K3DGalA
2-keto-3-deoxypentonic acids	2K3DPA
2-keto-3-deoxy-6-phosphogluconate	2K3D6PG
2-ketogluconate	2K
5-ketogluconate	5K
α-ketoglutarate	αKG
2-keto-6-phosphogluconate	2K6PG
DL-lactate	DL-lact
L-malate	L-mal
mannonic acid lactone	MA lact
mannose	M
methylene blue	M.B.
oxaloacetate	Ox
pentonic acids	PA
pentose	P
phenazine	phen
6-phosphogluconate	6PG
3-phosphoglyceraldehyde	3PGAld
3-phosphoglyceric acid	3PGA
phosphopyridine nucleotide (DPN or TPN)	PN
n-propionic acid	n-prop
pyruvate	Pyr
reduced DPN	DPNH
reduced TPN	TPNH
ribonic acid lactone	RA lact
ribose	R
ribose-5-phosphate	R5P
ribulose-5-phosphate	Ru5P
sedoheptulose-7-phosphate	Sed 7P
succinate	Succ
triphosphopyridine nucleotide	TPN
xylulose-5-phosphate	Xu5P

8. SUMMARY

The carbohydrate metabolism of *Pseudomonas* is reviewed and discussed. Much of our knowledge of the intermediary carbohydrate catabolism of the genus *Pseudomonas* is derived from the analysis of the enzymic equipment of *Pseudomonas saccharophila*, which is therefore reviewed as a model system. The acetic acid bacteria constitute another model system, because the enzymic constitution of many strains has been analyzed, and this has allowed a better insight into their natural relationships and possible phylogeny. The enzymic anatomy of the latter organisms is fairly well known, and by analogy a hypothesis on the enzymic anatomy of *Pseudomonas* cells is proposed.

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NOTES ADDED IN PROOF

1. Section 2 (a). Most of the assimilated material derived from pyruvate and giving rise to cell material has been shown to be poly-β-hydroxybutyric acid (Doudoroff, M. & Stanier, R. Y. (1959). Role of poly-β-hydroxybutyric acid in the assimilation of organic carbon by bacteria. *Nature, Lond.*, **183**, 1440).
2. Section 2 (d). Representatives of the genera *Escherichia*, *Aerobacter*, *Erwinia* and *Serratia* form inducible enzymes for a pathway leading from either D-mannonic or D-altronic acids to 2-keto-3-deoxygluconic acid and the corresponding 6-phosphoric ester, followed by a split into triose phosphate and pyruvate. In principle this mechanism is thus similar to the Entner-Doudoroff pathway. For a review see Rabinowitch, J. C. (1959). Fermentative metabolism. *Annu. Rev. Microbiol.* **13**, 441.
3. Section 5, Appendix 1. We have recently found that the culture tentatively classified as a saccharophilic *Alcaligenes faecalis* belongs to the genus *Agrobacterium* and that other species within this genus are capable of carrying out the same type of oxidation (Bernaerts & de Ley, unpublished data).

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DIFFERENTIAL TECHNIQUES AND METHODS OF ISOLATION OF *PSEUDOMONAS*

By K. KLINGE

Hygienisch-bakteriologisches Institut,
University of Erlangen, Germany

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1. Introduction

Bacteria belonging to the genus *Pseudomonas* occur almost everywhere in nature and can be isolated from most natural sources. The interest in (and also the classification of) *Pseudomonas* species stems from various groups of investigators: medical bacteriologists are concerned with *Ps. aeruginosa*, the human pathogenic species; plant pathologists study plant pathogenic *Pseudomonas* species (the species are named after the hosts and their pathogenicity is almost the only criterion for species differentiation); soil scientists and limnologists investigate the ecological importance of *Pseudomonas* spp. occurring in soil and water: biochemists are interested in the metabolic activities of particular *Pseudomonas* spp.; and both hygienists and food technologists are interested in preventing food deterioration by *Pseudomonas*, since the increasing use of refrigeration prevents deterioration by mesophilic bacteria, but leads to a selective growth of psychrophilic bacteria.

The study of *Pseudomonas* from so many different points of view has resulted in the use of different classifications, and it is quite possible that different investigators, working independently of each other and using different methods, examine the same *Pseudomonas* strains but name them differently (and *vice versa*) or classify as *Pseudomonas*, strains which do not belong to this genus. For example Elrod & Braun (1942) have shown the identity of *Ps. aeruginosa*, the human pathogen, with *Phytomonas polycolor*. the plant pathogen; and Clement & Gibbons (1960) demonstrated that certain cultures studied as *Pseudomonas* strains by biochemists belonged in fact to the genus *Aerobacter*. The failure to reach an agreement on the classification of *Pseudomonas* spp. and on the methods to be used in their identification is the result of the different attitudes of many investigators towards the characters to be used and the methods for their demonstration, and to the relative values of physiological and biochemical characters on the one hand and pathogenicity on the other. Gaby (1955) wrote of the situation. that "In many instances the species characteristics now listed are so vague and inadequate that laboratory re-identification of a culture that had lost its label would be impossible". and this situation has causes which must be considered in attempting to correct it; they are summarized below.

First. the biochemical properties are often tested by methods which do not take into account the particular metabolic pathways of these bacteria. For example, methods used for the determination of acid production from carbohydrates by Enterobacteriaceae are inadequate for *Pseudomonas*: the former attack carbohydrates by fermentation. the latter by oxidation. Second. no defined set of tests is applied to the examination of *Pseudomonas*: each worker examines those properties that seem most important to him. Third. with regard to *Pseudomonas*. Bergey's *Manual* (Breed. Murray & Smith. 1957), while paying respect to the historical description. does not list the results of modern test methods but gives only morphological characters and incomplete descriptions of biochemical behaviour. This applies also to the classification given by Krassilnikov (1959). The fact that the genus *Pseudomonas* in Bergey's *Manual* is divided into soil and water forms, sea water and brine forms, and plant pathogens complicates a classification which should be based on morphological and physiological characters and not on habitats. Fourth. some investigators speak only of green pigment producing. or fluorescent. bacteria to describe a group of organisms which have that single property in common. and sometimes also in order to avoid a more detailed examination of strains. Since it is well known that the ability to produce pigment can be lost. a system based solely on pigment production is of no value.

When the examination of *Pseudomonas* strains was begun it was intended neither to set up a new classification scheme or a new system for the more than 140 species listed in Bergey's *Manual*, nor to give a complete description of the genus *Pseudomonas*, a difficult task for a single worker. The problem was to classify certain *Pseudomonas* strains and, in some cases, to assess the similarity of two strains isolated from different sources. Two questions in particular drew my attention to *Pseudomonas*. Besides sporeforming bacteria only bacteria producing a diffusible green fluorescent pigment were found frequently in ground water. It was supposed by Knorr, Haas & Knorr (1956) that these micro-organisms must have escaped the predatory action of protozoa, to which the biological barrier in the soil is largely due. These authors showed that *in vitro* amoebae could take up and excrete spores without suffering any harm, whereas certain fluorescent bacteria killed and lyzed the protozoa *Amoeba limax*, *A. fluvialis*, *A. lacustris* and *Glaucoma pyriformis*. Other fluorescent bacteria did not kill protozoa, and it was concluded from these and other experiments that certain *Pseudomonas* strains produce substances toxic to protozoa. Later Gräf (1958) was able to isolate in crystalline form the antibiotic responsible for the antiprotozoal action. In view of these findings it seemed desirable to determine whether *Pseudomonas* strains which produce this antibiotic have any other common characteristics which would allow them to be rapidly differentiated from those which do not.

A similar problem arose from the fact that some refrigerated bottles containing blood for transfusion were found to contain *Pseudomonas*. The contamination could have occurred either before or during filling; but in some instances it probably occurred subsequently, when the bottles were opened. The bacteria introduced in this way multiplied during further storage in the refrigerator and the use of such infected blood caused severe and fatal reactions. In consequence it became necessary to determine whether a *Pseudomonas* strain isolated from the blood of a fatal case was the same as that obtained from the blood left in the bottle. Transfusion reactions due to contaminated blood or other liquids probably occur frequently, as indicated in the literature. Transfusion solutions contaminated by *Pseudomonas* have been described by Edsall & Wetterlow (1947), Braude, Sanford, Bartlett & Mallery (1952), Pittman (1953), Stevens *et al.* (1953), Bourgain, Bonnel & Raby (1958), Matthes, Orth & Köhler (1958) and Kaffka & Walther (1959). Further references to this subject can be found in the papers mentioned.

Because the descriptions of *Pseudomonas* given in the literature were insufficient, it was planned to study *Pseudomonas* strains isolated from blood, river water, ground water and clinical material, as well as stock strains, the main interest being in psychrophilic strains. The results of studies extending over several years are summarized below. It is believed that all the strains examined can be classified within three species of the genus *Pseudomonas*: *Ps. fluorescens*, *Ps. putida* and *Ps. aeruginosa*.

2. MORPHOLOGICAL CHARACTERS

(a) *Colonies*

Colonies of *Ps. fluorescens* and *Ps. putida* have no characteristic features. After incubation for 24 hr at 22° these species grow as circular convex smooth colonies with entire edges, the colony size increasing during further incubation. Rough forms also occur and have been found especially when the organisms were grown on agar media containing Tween 80 (Klinge, 1957*b*). The so-called pseudophage holes seen in cultures of *Ps. aeruginosa* on solid media have never been found in cultures of *Ps. putida* or *Ps. fluorescens*. Slimy, giant colonies were seldom found. These have been described by Romfeld (1956), Bornside & Richardson (1958) and Klinge (1959*a*). The suggestion made by Bornside & Richardson (1958) that such strains should be called *Ps. myxogenes* has not been followed since, as they also observed, this type of growth was not constant. The conditions necessary for the growth of giant colonies could not be established. In the temperature range 4–22° this type of growth occurred on nutrient agar, blood agar, egg-yolk agar and gelatin agar, irrespective of whether the plates were incubated in a dry or a humid atmosphere. The possibility that these strains might be infected by phages has not been investigated. Sonnenschein (1927) considered that the slimy growth of *Ps. aeruginosa* was caused by phages, and this problem has been discussed generally by Klinge (1957*d*) with regard to slime wall production. For the description of colony types exhibited by *Ps. aeruginosa* the reader is referred to the papers published by Gaby (1946, 1955) and to the photographs given therein.

(b) *Cells*

The organisms are Gram-negative, nonsporing, straight or sometimes slightly curved rods with polar flagella; the size of the rods depends upon the conditions of growth. *Ps. aeruginosa* has one polar flagellum, whereas *Ps. fluorescens* and *Ps. putida* have two to five. This was shown by Klinge (1959*a*) by electron microscopy and in smears stained by Leifson's method (Leifson, 1930) as described by Conn (1954*b*). The finding that the genus *Pseudomonas* includes bacteria with only polar flagella agrees with the description given by Migula (1897). Reports in the literature differ with respect to the number of flagella. Köhler (1957/58) and Gaby (1955) found lophotrichous flagella in *Ps. aeruginosa*, but later Gaby & Hadley (1957) stressed the typical monotrichous polar flagellation and this last observation is in accord with my own findings.

Capsules were never seen, although Villecourt & Jacobelli (1953) detected them in 11 out of 83 strains examined.

3. Biochemical Characters

(a) *Relation to oxygen*

Pseudomonas aeruginosa, *Ps. putida* and *Ps. fluorescens* are strictly aerobic organisms. When tubes of molten nutrient agar at 45° are inoculated, the inoculum well mixed and the tubes immediately cooled, incubation at a suitable temperature results in multiplication of *Pseudomonas* only on the surface. In tubes containing semisolid nutrient agar (0·3% agar), inoculated by needle, growth occurs only when the tubes are unsealed: when sealed with paraffin, at most only a trace of growth becomes visible (Klinge, 1959*a*). But ability to grow in the absence of oxygen depends on the presence of suitable hydrogen acceptors. Köhler (1957–58) described *Ps. aeruginosa* as being able to grow both aerobically and anaerobically, and this finding was probably due to the method he used. It is possible that strictly anaerobic conditions were not imposed; but also it may have been that his medium contained nitrate, as it is known that *Pseudomonas* strains which rapidly reduce nitrate to nitrite are able to use the former as a hydrogen acceptor (Fuchs, 1958*a*). In the procedure described by Fortner (1928), anaerobic conditions are not reached until *Ps. aeruginosa* has already grown.

(b) *Oxidative carbohydrate metabolism*

The *Pseudomonas* species mentioned attack carbohydrates by oxidation without direct phosphorylation. Glucose, for example, is oxidized to gluconic acid and then to 2-ketogluconic acid. No gas is produced. The important literature on results obtained by the use of manometric methods applied to dried and ultrasonically treated cells has been summarized briefly by Klinge (1959*a*). This pathway of carbohydrate metabolism yields a small amount of a weak acid, but the production of this may be masked by alkalinity resulting from the decomposition of the peptone in the medium. Hugh & Leifson (1953) therefore devised a medium and a test method which permits the detection of acid production and the differentiation of oxidizing bacteria from those which ferment or do not attack carbohydrates. The medium has a low peptone and a high carbohydrate content. Two tubes of this are inoculated; one is layered with paraffin to prevent the access of oxygen and the other is not. Organisms which have no action on carbohydrates do not change the pH indicator, oxidizing bacteria change the colour in the open tube only and produce no gas, whereas fermentative organisms produce acid, or acid and gas, in both tubes. The production of alkali and gas from glucose, mannitol and glycerol, described by Krassilnikov (1959) for *Pseudomonas*, is not believed to be correct.

In the present tests on about one hundred *Pseudomonas* strains acid (but never gas) was formed in the open tube only. The medium contained (all as % w/v): trypsin digested peptone (Merck), 0·2; NaCl, 0·5; KH_2PO_4, 0·03; agar, 0·3; bromothymol blue (in water), 0·003; and carbohydrate, 1·0; pH 7·1. The medium was tubed in 4·0 ml quantities. It has since become commercially available (Difco O/F medium).

Neisseria winogradskyi (Villecourt & Jacobelli, 1954), *Bacterium anitratum* (Hugh & Leifson, 1953; Villecourt & Jacobelli, 1954; Klinge, 1959*d*), *Moraxella lwoffi* var. *glucidolytica* and *Diplococcus mucosus* (Villecourt & Jacobelli, 1954; Klinge, 1959*d*) belong to the oxidizing group. Villecourt & Blachère (1955) have shown that this group cannot utilize lactose as a source of carbon for growth but do convert it to lactobionic acid, which is not metabolized further. With some *Pseudomonas* species, other than those described here, Bentley & Slechta (1960) reported the oxidation of mono- and disaccharides to aldonic acids. Despite the fact that these organisms are no longer listed in Bergey's *Manual* they should be included in the genus *Achromobacter*. They can be distinguished from *Pseudomonas*, *Flavobacterium* and *Brucella* by the type of flagellation (Leifson, 1958) and from *Pseudomonas* and *Brucella* by their negative oxidase reaction.

Pseudomonas strains which attack carbohydrates may also be recognized by their production of 2-ketogluconic acid, which is a natural chelator (Duff & Webley, 1959). The gluconate oxidation test used by Haynes (1951) and by Gaby & Free (1953) is a useful tool for the identification of *Pseudomonas*, but is not specific for this genus. Stewart (1959) described the production of 5-ketogluconic acid from glucose by *Pseudomonas*.

The medium devised by Hugh & Leifson (1953) for demonstrating acid production from carbohydrates is superior to the synthetic media employed by Stein, Weaver & Scherago (1942), Elrod & Braun (1942) and Salvin & Lewis (1946), which contained carbohydrate as the only available source of carbon and only inorganic nitrogen. Liu (1952) showed that in such media *Ps. aeruginosa* utilizes fewer carbohydrates than in peptone media. Some workers add 10% of carbohydrate to media with a high peptone content, but this method is very expensive. Rhodes (1959) demonstrated that many strains of *Ps. fluorescens* are able to grow in synthetic media containing no carbohydrate. Media employed for the demonstration of carbohydrate utilization must therefore be properly checked.

Pseudomonas strains also occur which do not attack carbohydrates at all. Hugh & Ryschenkow (1960) described such physiologically 'alcaligenes-like' bacteria as *Ps. multophila* n. sp. and Nyberg (1934–5) found similar lophotrichous organisms which he named *Ps. alcaligenes*, separating them from the peritrichous organisms named at that time *Bacillus faecalis alcaligenes* Petruschky.

From the above it is logical to conclude that acid production from carbohydrates cannot be determined in media with a high ratio of peptone to carbohydrate. Bender & Levine (1942) showed that in 35% of the *Pseudomonas* strains examined the acid produced in such media was masked by alkali production from the peptone.

Table 1. *Acid production by three* Pseudomonas *spp. from sugars, glucosides and alcohols in agar slants containing* 2% *of carbohydrate and* 0·1% *of peptone*

Data from Klinge (1959*a*)

Carbon source	Percentage of strains positive in species		
	aeruginosa (at 37°)	*fluorescens* (at 22°)	*putida* (at 22°)
Mannose	100	100	100
Glucose	100	100	100
Galactose	100	100	100
Fructose	(100)*	(100)	(100)
Arabinose	100	100	100
Xylose	100	100	100
Mannitol	(73)	(100)	(100)
Glycerol	(100)	(100)	(100)
Trehalose	(93)	(52)	(83)
Inositol	0	(63)	(67)
Rhamnose	0	(81)	(83)
Sorbitol	0	(70)	(83)
Sucrose	0	(52)†	(33)†
Maltose	0	0	0
Lactose	0	0	0
Dulcitol	0	0	0
Adonitol	0	0	0
Salicin	0	0	0
Starch	0	0	0

* Brackets indicate late acid production (after 4–5 days). † All strains produced levan.

According to Véron & Chatelain (1960), acid production from carbohydrates occurs earlier and with more strains in meat infusion agar than in peptone agar.

Following a suggestion made by Dr. P. Liu (University of Louisville) tests were made for acid production in agar slants: moreover the concentration of the carbohydrate was increased to 2% and that of the peptone reduced to 0·1%. The cultures were incubated at the appropriate temperature for 14 days, with the results summarized in Table 1.

The finding of Gaby (1955) that no *Ps. aeruginosa* strains produce acid from sucrose has been confirmed; but this observation is not valid for *Pseudomonas* strains which form levan from sucrose (Klinge, 1959*a*) since these strains oxidize the glucose which is liberated. No levan producing *Ps. aeruginosa* strains have been encountered, but 29% of the *Ps. fluorescens* strains and 8% of the *Ps. putida* strains tested did so (Klinge, 1958, 1959*a*) and hence produced acid from sucrose. Our results with *Pseudomonas* agree with those summarized by Dowson (1939), except that we did not find strains which could attack starch. Otherwise there is no known biochemical distinction between the plant pathogenic and the nonpathogenic types (Erikson, 1945; Dowson, 1939; Klinge, 1959*a*).

(c) *Levan production*

Levan is produced by *Ps. fluorescens* (Fuchs, 1956; Klinge, 1958), *Ps. putida* (Klinge, 1959*a*), *Ps. aureofaciens* (Fuchs, 1956), *Ps. prunicola* (Bell & Dedonder, 1954), *Ps. chlororaphis* (Fuchs, 1956) and other plant pathogenic *Pseudomonas* strains (Cooper & Preston, 1935). Levan production can easily be demonstrated by allowing the organisms to grow on nutrient agar containing 4% of sucrose. The levan synthesizing system is intracellular. It should be mentioned that Ørskov (1958) described the interesting phenomenon of growing polysaccharide colonies which were devoid of cells. It was assumed by the author that after the cells of a colony had swollen and burst, the free enzyme system synthesized levan on the same site. The levan produced looks like a colony.

Levan producing *Pseudomonas* strains, when grown on agar containing 4% of sucrose, are completely resistant to the predatory action of amoebae (Klinge, 1958) and to chloroform vapour (Klinge, 1959*b*).

Since levan production has been discussed by Fuchs (1958*a*,*b*) in connection with the pathogenicity of bacteria for plants and the mechanism of plant disease, mention may also be made of the production of pectinase by *Pseudomonas* strains (Paton, 1958), which has also been suggested as a factor in pathogenicity. The extracellular slime production of highly virulent *Ps. solanacearum* has also been considered as a factor in pathogenicity. The slime was identified by Dudman (1959) as a mixture of polysaccharides (no levan) and relatively simple peptides.

(d) *Oxidase reaction*

With regard to the application of the oxidase test for the identification of *Pseudomonas* strains, Gaby & Hadley (1957) discussed the mechanism of the reaction and devised a new method. Klinge (1959*a*) reviewed the modifications of this method and discussed the results obtained by their use. Schultze (1910), Kramer (1912), Loele (1929), Frei, Riedmüller & Almasy (1934), Gordon & McLeod (1928), Kovacs (1956), Gaby & Hadley (1957), Gaby & Free (1958) and Klinge (1959*a*) obtained a positive oxidase or cytochrome oxidase reaction with *Pseudomonas*. Lutz, Schaeffer & Hofferer (1958) and Köhler (1959) obtained negative results in some cases and felt that the test was not reliable.

In view of these results it was decided to reinvestigate the problem (Klinge, 1960) and, like the authors mentioned, the method recommended by Gaby & Hadley (1957) was used. This consists in the addition of 0.5 ml of the reagent (2 parts of 1·0% α-naphthol in 95% ethanol + 3 parts of 1% dimethyl-*p*-phenylenediamine oxalate in water) to 4·5 ml of an 18–24 hr liquid culture. It was found that the results depended on the strains of bacteria, on the nutrient broth and on the reagents. Köhler (pers. comm.) used an amorphous dimethyl-*p*-phenylenediamine oxalate prepared in his laboratory and found that the crystalline substance prepared from it gave the same results as the commercially available (Difco) product used by Gaby & Hadley (1957). After trying the various reagents listed in Table 2 and obtaining different results, depending on the reagent and on the medium, the use of dimethyl-*p*-phenylenediamine oxalate is recommended as the most stable and least toxic of the three substances examined. The blue colour developed with tetramethyl-*p*-phenylenediamine dihydrochloride faded very quickly, and the highest number of positive reactions was obtained if the results were read in the first minute after adding the reagents. It is believed that the negative results obtained by Lutz *et al.* (1958) can be explained by their use of an unsuitable medium, although the recipe was not given. The results set out in Table 2 demonstrate that the differences are due to the media, and an attempt was made to find out why this was so. It was found that after 18–24 hr incubation

Table 2. *The influence of reagents and culture media on the results of the oxidase test performed according to Gaby & Hadley* (1957) *and applied to* Pseudomonas aeruginosa, Ps. fluorescens *and* Ps. putida

Medium	Data from Klinge (1960) Percentage of strains giving a positive reaction (A) immediately and (B) 3 min after reading					
	Dimethyl-*p*-phenylenediamine dihydrochloride		Dimethyl-*p*-phenylenediamine oxalate		Tetramethyl-*p*-phenylenediamine dihydrochloride	
	A	B	A	B	A	B
Meat infusion broth + 1% peptone	100	100	82	100	94	52
Meat infusion broth + 1% peptone + 1% glucose	42	42	61	58	73	39
Cenovis yeast extract broth + 1% peptone	27	88	64	73	67	42
Liebig's meat extract broth + 1% petone	42	52	58	73	58	39

Pseudomonas cultures have a pH within the range 6–8, and in this range pH does not influence the oxidase reaction. The reaction was then examined in various *Pseudomonas* cultures after the addition of reducing agents, and this enabled the strains to be divided into two groups. The first group gave a positive reaction in meat infusion broth but not in the other media listed in Table 2, and the second group gave positive reactions in all the media. The cultures of the first group had a stronger reducing power than those of the second group, as measured by the methylene blue reduction test, and their oxidase reactions were inhibited by lower concentrations of reducing agents than were required by the second group. It is necessary to stress these facts, since they indicate the importance of checking whether the media used in this test are suitable.

When the reagent is dropped on to colonies grown on the surface of solid media the composition of the medium does not influence the oxidase reaction. The procedure described by Kovacs (1956) consists in smearing the bacteria to be tested on filter paper previously soaked with the reagent: but as also found by Gaby & Free (1958) this method is too sensitive, probably because of the rapid autoxidation of the reagent.

It was observed that the time required for the development of the blue colour varied from seconds to minutes. It is considered that the differentiation of *Alcaligenes* from *Pseudomonas* by evaluating the rate of colour development, as reported by Gaby & Hadley (1957), is not possible: to make the differentiation certain determination of the nature of the carbohydrate metabolism with the O/F medium and staining for flagella are needed. From the data given by Gaby & Hadley (1957) it cannot be excluded that certain strains designated *Alcaligenes* by them were in fact *Pseudomonas*, as they had polar flagella. The flagella stain and the use of the O/F medium are also necessary to differentiate between the oxidase positive genera *Vibrio*, *Pseudomonas* and *Aeromonas*. We have no experience of the genus *Lophomonas*. With respect to *Alcaligenes* the reaction time should not be overemphasized.

(e) *Pigment production*

The names of the three species discussed are derived from the pigments produced by them. It is well known that pigment production is influenced by the composition of the medium, but the media devised by King, Ward & Raney (1954) have proved very useful. That for production of the fluorescent pigment has a high phosphate content, and this inhibits pyocyanin production; accordingly the medium which stimulates pyocyanin production has a low phosphate content. Since both media contain specially recommended peptones which were not available their composition was modified (Klinge, 1959*a*). Pyocyanin or pyorubrin, as well as the fluorescent pigment, were readily produced after 24 hr incubation. Traces of the fluorescent pigment can be detected by viewing the cultures in UV light. Martineau & Forget (1958) demonstrated the suitability of Sabouraud's maltose agar for pyocyanin production.

For production of the fluorescent pigment Paton (1959) recommended the use of a medium containing inorganic salts, with gluconate as carbon source, after treatment with 8-hydroxyquinoline. He considered the low content of available iron to be primarily responsible for stimulating pigment production.

Since it is known that the ability to produce either one or both pigments can be lost after subculturing (Wilson & Miles, 1955), unpigmented strains are not to be excluded from any of the three species provided the other morphological and biochemical properties are characteristic (Gaby, 1955; Klinge, 1959*a*). Over several years strains which have lost the ability to produce pigment have been encountered: these have proved not to be different from the original strains with respect to the lysotype and other biochemical characters. It should be mentioned that pigment production by *Pseudomonas* strains can be evaluated only when media optimal for pigment production are used.

(f) *Growth-temperature relationship*

According to Wilson & Miles (1955) *Ps. fluorescens* grows between 0° and 37° and *Ps. aeruginosa* between 5° and 42°, the optimum temperatures being 25° and 37° respectively. The value of determinations of growth rate at different temperatures depends on the method used. Conn (1954*a*) recommended that the type of medium used should be stated and that cultures should be tested at 5° intervals, and this is very reasonable. It is our experience that only liquid media (because of the better distribution of bacterial clumps and more rapid attainment of temperature) are suitable. Using thermocouples, the temperatures of various solid and liquid media were taken before and during incubation in a water bath and in an incubator, to discover the rate of temperature equilibration. From the results obtained (Table 3) it is clear that only the liquid media incubated in a water bath fulfilled the requirements for accurate determinations.

Table 3. *The effect of medium and incubation conditions on the time taken by cultures to reach equilibrium temperature*

Data from Klinge (1959*a*)

Medium	Method of incubation	Temperature* (°C) Initial	Temperature* (°C) Final	Difference (°C)	Time (min) for equilibration
Nutrient broth	In tubes in a water bath	21·0	36·5	15·5	7·5
Nutrient broth	In tubes in an incubator	24·5	35·7	11·2	77·5
Nutrient agar	In Petri dishes in an incubator	22·0	35·8	13·8	180

* Measured by thermocouples.

Under optimum conditions (liquid cultures in a water bath) it was found that the size of the inoculum was also important. Most *Pseudomonas* strains produce in liquid culture a pellicle or aggregation of cells at the surface, and if this is used as inoculum a relatively large mass of clumped, slimy bacterial substance is transferred. From such an inoculum most *Pseudomonas* strains grew at 37°; but when a drop of a 24 hr broth culture diluted 1:100 was used no strain of *Ps. fluorescens* or *Ps. putida* grew at 37°, whereas all *Ps. aeruginosa* strains grew at 42°. These results are in accordance with those of Ingraham (1958) who used synchronous cultures and found the shortest generation time of *Ps. fluorescens* to be 51 min at 32° and that of *Ps. aeruginosa* to be 22 min at 40°, while the lowest temperatures at which no growth occurred were 36° and 46° respectively. It is therefore believed that ability to grow at 42° is a valid criterion for the identification of *Ps. aeruginosa* and that failure to grow at 37° or 38° is sound for the identification of *Ps. putida* and *Ps. fluorescens*. The ability of *Ps. aeruginosa* to grow at 42° was a criterion stressed by Haynes (1951).

Thermal inactivation tests performed with *Ps. fluorescens* confirmed our results: 24 hr cultures diluted 1:5 and 1:100 were killed at 37° within 48 and 24 hr, respectively.

(g) *Lecithinase*

During investigations on proteolysis and haemolysis by various bacteria (Klinge, 1957*c*) zones of precipitation were observed around colonies of *Ps. fluorescens* grown on nutrient agar containing egg yolk, similar to the egg yolk reaction given by *Serratia marcescens* (Monsour & Colmer, 1952). At that time we were not aware that Villecourt & Jacobelli (1953) had found that the egg yolk reaction was given by some *Pseudomonas* strains. Those authors did not investigate the mechanism of the reaction; but our quantitative experiments led to the conclusion that *Ps. fluorescens* causes the egg yolk reaction by means of lecithinases C and D, because free choline and an increased amount of acid soluble phosphorus were found (Klinge, 1957*b*). The zones of precipitation differed in extent, and after having tested many strains qualitative differences, whose cause could be traced to the composition of the medium, were also found. The egg yolk reaction of some *Pseudomonas* strains was inhibited by peptone, by alkalinity, or by calcium acetate at a concentration higher than 0·005 M, and it is therefore recommended that glucose-phosphate agar without peptone, and saline containing 5% egg yolk, be used for the lecithinase test with *Pseudomonas*. This inhibitory action of peptone is the opposite of the stimulatory effect found with *Serratia plymuthicum* (Klinge, 1957*a*). *Ps. putida* did not give the egg yolk reaction, nor did it liberate acid soluble phosphorus or choline from egg yolk. *Ps. aeruginosa* liberated as much acid soluble phosphorus and choline from egg yolk as *Ps. fluorescens* but gave no egg yolk reaction. Colonies of *Ps. aeruginosa* on egg yolk agar are flat and spreading and pyocyanin production is stimulated, and it is thus possible to differentiate the three species of *Pseudomonas* from each other by means of their behaviour on egg yolk agar.

Supernatants from centrifuged broth cultures of *Ps. fluorescens* caused turbidity when added to diluted and filtered egg yolk, and the increase in turbidity with time followed a first order reaction. The highest value for the reaction constant was obtained at 45°. No turbidity and no liberation of acid soluble phosphorus occurred after heating the supernatant for 20 min at 60° or above.

(h) *Lipase*

Most strains of *Ps. fluorescens* and *Ps. aeruginosa* possess lipase, as may be demonstrated by means of the Nile blue sulphate technique (Klinge, 1956) with olive oil or egg yolk fat as substrate. As Nile blue sulphate is toxic towards some strains lipase action is now demonstrated by the production of fatty acid globules around colonies grown on nutrient agar containing Tween 80. The extensive studies of Sierra (1957) have proved the usefulness of this method. The peptone content of the medium did not affect the lipolytic activities of the *Pseudomonas* strains in the Tween 80 method, though it did do so with the Nile blue sulphate technique.

(j) *Gelatinase*

Pseudomonas fluorescens and *Ps. aeruginosa* but not *Ps. putida* hydrolyzed gelatin, as shown by the technique described by Frazier (1926), which consists in flooding inoculated and incubated plates of nutrient agar containing 0·4% of gelatin with a solution of mercuric chloride in dilute hydrochloric acid. Gelatinase action is recognized by zones where precipitation of the gelatin does not occur. Great attention is no longer paid to the type of liquefaction of gelatin in tubes, since this varies with the type, consistency and age of the gelatin medium. The diffusion of oxygen into the medium probably determines the type of liquefaction caused by these strictly aerobic organisms.

(k) *Nitrate reduction*

Pseudomonas aeruginosa reduced nitrate to gaseous nitrogen, and some strains of *Ps. fluorescens* and *Ps. putida* produced nitrite from nitrate in 3–4 days. Reduction of nitrate by the other strains could be found only when the strains were passaged through a synthetic medium containing nitrate as the only source of nitrogen (Diminick, 1947). In every case nitrite was found when a modification of the rapid method of Brough (1950) modified by Clarke & Cowan (1953) was applied. This was performed by incubating 0·2 ml of a suspension prepared by suspending the growth from one agar slant in 1·0 ml of 1·0 M/15 phosphate buffer containing 0·1% KNO_3 at pH 6·8 for 15–30 min. Strains which gave a weak nitrate reduction reduced nitrite very rapidly.

(l) *Production of a haemolysin toxic to protozoa*

In contradistinction to Liu (1957) haemolytic *Ps. fluorescens* strains have been found (Klinge, 1956, 1957*b*) which did not digest haemoglobin in the test methods used. In order to avoid the nonspecific haemolysis often observed after prolonged incubation the organisms are inoculated on to agar containing 2% human blood layered thinly on normal nutrient agar plates. All haemolytic strains produce a clear zone of haemolysis not later than 48 hr after inoculation. Villecourt & Jacobelli (1953) found a large number of haemolytic strains (68 out of 83).

The haemolysis is caused by a substance which is toxic to protozoa (Klinge & Gräf, 1959). Twenty μg of the isolated haemolysin dissolved in 1·0 ml of normal saline caused 50% lysis of 1·0 ml of 1% washed human red cells in 60 min at 37°. The haemolysin is an anionic surface active agent which at a concentration of $1:10^5$ lowers the surface tension of 0·85% NaCl of pH 7·0 to 32 dynes/cm^2. The haemolytic activity is inhibited by serum while the haemolysin inhibits the egg yolk reaction. The concentration-time curves for the lysis of red cells and amoebae are parallel.

Only the haemolytic strains of *Ps. fluorescens* lyze amoebae within 5–10 min when inoculated near the bacterial growth on agar plates. All strains of *Ps. aeruginosa* were haemolytic. Some strains also lyzed amoebae, but no active substance could be isolated by the technique used for the isolation of the antibiotic compound produced by *Ps. fluorescens* (Gräf, 1958). Up to now no attempt has been made to isolate from natural sources (surface water heavily contaminated by bacteria, sewage, etc.) the antiprotozoal substance produced by *Ps. fluorescens.* Haemolysin production is by no means a constant property; most strains of *Ps. fluorescens* lose the ability during subculture but without changing lysotype.

(m) *Other characters*

Litmus milk, used as a test medium in former studies, has been found to give irregular results, due apparently to the varying quality of milk before and after preparation, and it is no longer employed.

Table 4. *The behaviour of three* Pseudomonas *species in further biochemical tests*

Data from Klinge (1959*a*)

Test		Reactions* of species		
		aeruginosa	*fluorescens*	*putida*
Catalase		+	+	+
Methyl red		—	—	—
Acetylmethylcarbinol		—	—	—
Indole		—	—	—
Urea		—	—	—
Hydrogen sulphide		V	—	—
KCN		+	+	+
Utilization of NH_4^+ with	glucose	+	+	+
	citrate	+	+	+
Oxidative deamination of β-phenylalanine		—	—	—

* Reactions: +, positive; —, negative; V, very weak.

The determination of ability to effect the oxidative deamination of phenylalanine has been included, because Meyer, Malgras, Romond & Boeglin (1960) found this test positive with *Ps. fluorescens*; but their results could not be confirmed. The other tests (Table 4) are performed according to the standard methods used for the determination of the biochemical properties of members of the Enterobacteriaceae.

4. Principles of Selective Media

(a) *Media containing a selective carbon source*

It is possible that the ability of a bacterial species to utilize selectively for growth a defined nutrient could be used determinatively. The necessary criteria are that the nutrient must be specifically utilized by one species and that no other nutrient must be introduced which can replace it. Under these conditions a medium could be developed for estimating the number of organisms of a single species present in a sample of soil or water free from nutrients. Villecourt, Blachère & Jacobelli (1952) have shown that in the presence of nitrate as nitrogen source and β-hydroxybutyrate or acetoacetate as carbon source *Pseudomonas* could be isolated from soil; but in every experiment *Neisseria winogradskyi* also grew. Factors limiting this method are: that probably there will be no nutrient utilized solely by all strains of only one bacterial species, and that other nutrients must not be introduced. Therefore this method would never work for the estimation of *Pseudomonas* in samples of food.

(b) *Media containing cationic compounds*

Apart from pyocyanin, used by Ringer & Drake (1952) to inhibit bacteria other than *Ps. aeruginosa*, cationic compounds have mainly been used. For the selection of *Ps. aeruginosa* Habs & Kirschner (1943) used $1:10^5$ malachite green, Winkle (1955) used $2:10^4$ brilliant green and Lowbury (1951) used $1:10^3$ cetyltrimethylammonium bromide. None of these substances was used for differentiating between *Pseudomonas* species, but for the inhibition of all organisms other than *Ps. aeruginosa*. Trials of these media too have shown that differentiation of *Pseudomonas* species was not possible (Klinge, 1959*a*).

Selenka (1958) developed media containing $1:10^2$ and $2:10^3$ triphenyltetrazolium chloride (TPTC) and showed that at 37° and 42° only *Ps. aeruginosa* grew. By this means it was possible to differentiate between mesophilic and psychrophilic organisms, between *Ps. aeruginosa* and *Ps. fluorescens* as well as *Ps. putida*. Selenka (1960) applied his method to the quantitative determination of *Ps. aeruginosa* in surface waters. By using meat infusion-peptone agar, yeast extract-peptone agar and the pyocyanin medium of King, Ward & Raney (1954), each containing $2:10^3$ TPTC, he showed that very small inocula of pure cultures of *Ps. aeruginosa* would grow. In practical use this medium was also valuable. Water samples were plated on nutrient agar containing 1% of peptone and $2:10^3$ TPTC, incubated for two days at 37° and the plates then flooded with the oxidase reagent. Blue colonies were counted and picked off. From a sample containing 2,653 viable bacteria, 89 oxidase positive colonies were counted, 62 of which could be identified as *Ps. aeruginosa*. Despite the fact that all the colonies which grow on this medium are red coloured due to the production of the formazan, the blue coloured oxidase positive colonies are easily recognized. By means of this method between 2 and 7 *Ps. aeruginosa* were found/ml of river water.

5. ANTIBIOTIC ACTIVITY

Besides the antibiotic activity exhibited by haemolytic *Ps. fluorescens* strains towards protozoa, haemolytic and nonhaemolytic strains of the same species developed an antibiotic activity against Gram-negative and Gram-positive organisms. This has been shown by Frost (1904), Olitsky (1891) and Lewis (1929), also by Klinge (1959*b*) who performed the tests according to the method of Frédéricq & Levine (1947) as modified by Kjems (1955). The same method was applied to the examination of antibiotic interrelationships within the species *Ps. fluorescens*. Some strains possessed great activity and the results have been constant and reproducible. By means of the spectrum strains could be compared. The supernatant from a broth culture of *Ps. fluorescens* was shown not merely to inhibit sensitive strains of *Ps. fluorescens* but to kill them, though the bactericidal effect depended on the number of sensitive bacteria exposed. The active substance was not destroyed by boiling for 5 min or by autoclaving for 20 min at 120°.

6. USE OF PHAGES FOR THE IDENTIFICATION OF STRAINS

The antibiotic spectrum of *Ps. fluorescens* strains against each other did not help greatly in identifying two strains. Phages were isolated in order to determine the lysotype (Klinge, 1959*c*). It is not necessary to refer to the many papers on *Ps. aeruginosa* phages nor to recall all the results obtained by us with *Ps. fluorescens* and *Ps. putida* phages, but the results may be summarized as follows. All phages isolated were species specific, but no phage was found to lyze all strains of the homologous species when tested in 1× or 100× the routine test dilution (which is the highest dilution giving complete lysis of the host strain). The identity of two strains which differed with respect to acid production from trehalose, production of haemolysin and production of fluorescent pigment was demonstrated.

7. SEROLOGY OF *PSEUDOMONAS*

Besides the many early investigations Habs (1957) and Kleinmaier (1957) recently examined *Ps. aeruginosa* strains by agglutination (O antigen) and Köhler (1957) performed precipitation tests using formamide extracts. The two groups of workers established a serological scheme which permits one to follow up the occurrence and the spread of *Ps. aeruginosa*. Kleinmaier & Quincke (1959) and Köhler (1957) examined strains of human origin, Sandvik (1960) and Köhler (1960) strains of animal origin.

8. DISCUSSION

The genus *Pseudomonas* comprises Gram-negative, polarly flagellated rods which either do not attack carbohydrates or attack them oxidatively. Using our experimental work as a basis, we can divide the strains examined by us into three species by using the differential criteria set out in Table 5.

There seems to be no justification for dividing the strains examined into groups and subgroups, as was done by Seleen & Stark (1943) who examined only those strains which produced pigment. It has been found that typical *Ps. fluorescens* strains can lose the ability to produce pigment without apparently changing any other biochemical and morphological character or the lysotype, and accordingly strains are not divided into those which produce pigment and those which do not, a differentiation made by Shewan, Hodgkiss & Liston (1954) on the basis of differing resistance to antibiotics. It is considered that a scheme of classification based only on different sensitivity to various antibiotics cannot be generally valid, because it is known that many strains can be 'trained' to become resistant. The scheme devised by Shewan *et al.* (1954) may be valid for the classification of bacteria isolated from sea water, but it is not valid for strains isolated from human beings treated with antibiotics. Strains of *Diplococcus mucosus* (*Achromobacter*) isolated by Legler (pers. comm.) were tested for sensitivity to streptomycin, and out of 73 strains 10 were sensitive to 12–50 μg and 63 to 100–2,500 μg. Also, out of 70 strains 45 were sensitive to 31–62 μg of chloramphenicol and 25 to 125–250 μg. This means that half of the strains would not be *Achromobacter* because they are resistant to these antibiotics.

Table 5. *The differentiation of* Ps. aeruginosa, Ps. fluorescens *and* Ps. putida

Differential criteria		Characters and reactions* of the species		
		fluorescens	*putida*	*aeruginosa*
Flagella		Polar, multitrichous	Polar, multitrichous	Polar, monotrichous
Growth at	22°	+	+	+
	37°	—	—	+
	42°	—	—	+
Pigment	pyocyanin	—	—	+
	fluorescent	+	+	+
Egg yolk reaction		+	—	—
Gelatin liquefaction		+	—	+

* Reaction: +, positive; —, negative.

Seleen & Stark (1943) divided their strains into groups and subgroups according to behaviour in the following tests: growth at 5° and 42°, action on milk, liquefaction of gelatin, reduction of nitrate, production of pyocyanin, utilization of sucrose, acetic acid, lactic acid and tartaric acid and growth in the presence of phenol, sodium cyanide and sodium chloride. Villecourt, Blachère & Jacobelli (1952) divided their strains into groups and subgroups on the following characters: gelatin liquefaction, gas from nitrate, nitrite from nitrate and acid from sucrose and maltose. With respect to the establishment of groups it is felt that it is not advisable to divide strains into groups on the basis of properties whose constancy is uncertain. As we have shown, all the *Pseudomonas* strains examined can be trained to reduce nitrate. A differentiation based on gelatin liquefaction separates proteolytic from non-proteolytic strains, and we agree with this separation and would suggest that the group of levan producing strains should also be separated. This group would coincide with the group 'sucrose utilizers' of Seleen & Stark (1943) and the 'producers of acid from sucrose' of Villecourt, Blachère & Jacobelli (1952). With respect to our material there is no further classification possible. It would help very much if in all investigations concerned with *Pseudomonas* the characters of the genus as discussed in this paper were to be established at the outset.

Moore & Rickett (1960) have shown how, with a small series of tests, *Pseudomonas* strains may be shown to be such and distinguished from *Achromobacter*. Rhodes (1959) showed that the 165 strains designated *Ps. fluorescens* by her were quite heterogeneous; but not all the tests which she employed in demonstrating this heterogeneity are criteria commonly used for species differentiation.

Sherris *et al.* (1959) and Thornley (1960) have shown that the enzyme system involved in the decomposition of arginine is possessed by all *Pseudomonas* examined and allows, with very rare exceptions, a differentiation of *Pseudomonas* from other bacteria.

9. Summary

A short review is given on the identification and isolation of *Ps. aeruginosa*, *Ps. fluorescens* and *Ps. putida*. This report is based mainly on experiments performed by the author. The work was carried out with the aim of identifying psychrophilic *Pseudomonas* species. Before any groups or subgroups in the highly confused scheme of classification of *Pseudomonas* can be established the strains should conform with regard to the following characters: Gram-negative, nonsporing rods with polar flagella; strictly aerobic; carbohydrate metabolism, if any, oxidative; positive catalase reaction; positive cytochrome oxidase reaction; growth in ammonium citrate; KCN positive; methyl red negative; acetylmethylcarbinol negative; urease negative; indole negative. Further tests should include gelatin liquefaction, pigment production, growth temperature limits, gluconate oxidation, nitrate reduction, production of acid from glucose, arabinose, xylose, mannose and galactose, no acid production from lactose, dulcitol, adonitol or salicin. When these tests and eventually others also are applied to *Pseudomonas* strains, plant pathogens should also be examined. When the identity of two strains is being investigated the determination of the lysotype is recommended since this has proved very useful.

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The present address of the author is the Institut für Hygiene und Mikrobiologie, Universität des Saarlandes, Landeskrankenhaus Bau 5, Homburg (Saar), Germany.

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper V)

THE *PSEUDOMONAS* AND *ACHROMOBACTER* GROUPS OF BACTERIA IN THE SPOILAGE OF MARINE WHITE FISH

By J. M. SHEWAN, G. HOBBS and W. HODGKISS
Torry Research Station, Aberdeen

SUMMARY: Total colony counts and analyses of the bacterial flora of the skin slime of cod, carried out at 5-day intervals during the stowage of the fish in ice, showed that organisms of the genera *Pseudomonas* and *Achromobacter* became predominant in the later stages of spoilage.

In this country about half of the white fish landed at ports is brought by distant water trawlers which operate in Arctic waters north of Norway and around Iceland and Greenland (Fig. 1). As these ships have an average round trip of 18–22 days the fish, when landed at the ports, has been in ice for 5–15 days, occasionally longer. Ships fishing on grounds nearer home usually average less than 7 days for a voyage, and accordingly the spoilage problems are much less acute. The fish are caught in a trawl net which is towed along the sea bed for 2–4 hr and then hauled on to the deck. The fish are eviscerated, washed and stored in ice in the fish room until landed at the port.

It is well established that the putrefactive odours and flavours produced during stowage are the results of bacterial activity. The bacteriology of fresh and spoiling marine fish has been studied by many workers, including Hunter (1920*a*,*b*; 1922*a*,*b*), Fellers (1926), Sanborn (1930), Schönberg (1930), Stewart (1932), Wood (1940), Shewan (1942, 1944), Aschehoug & Vesterhus (1943, 1947), Thjøtta & Sømme (1943), Dyer (1947) and Shewan & Liston (1956). Much of this work has been reviewed by Shewan (1949, 1960) and Tarr (1954). Most of these investigations demonstrated that the bacteria predominating on fresh and spoiling fish were psychrophilic Gram-negative rods. In view of the radical changes that have taken place in bacterial classification in recent years (see, for example, Ingram & Shewan, 1960), it is almost impossible to assess the true taxonomic position of many of the bacteria described by the earlier workers, especially before 1948. The results to be presented here show the typical changes in the bacterial flora of white fish during spoilage. The experiments were part of an investigation into the reasons for the differences in the keeping qualities of fish from different fishing grounds and at different times of the year, which for many years have been known by the trade to exist.

Materials and Methods

The cod (*Gadus morrhuae*) used in these experiments were caught with a trawl net by the R.V. 'Sir William Hardy'. The results to be presented were obtained on two expeditions, one in June 1959 and one in September 1959. On each voyage fish from three fishing grounds, North Cape (Barents Sea), Faroes and North Sea, were sampled. The first of these grounds yields a race of cod which travel considerable distances to the spawning grounds, the so-called 'oceanic' cod; whereas the Faroes and North Sea grounds yield 'coastal' cod, which spawn relatively close to their normal feeding grounds.

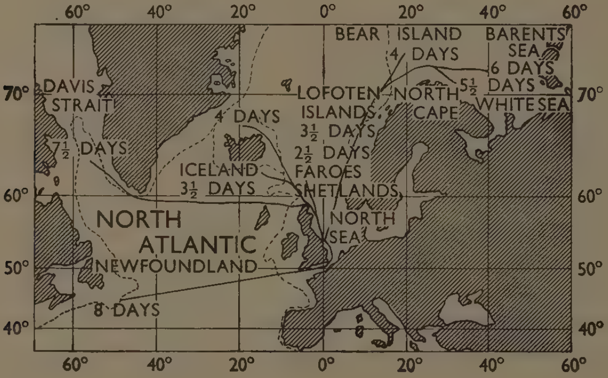
60°
40°
20°
0°
20°
40°
60°
70°
60°
50°
40°
DAVIS STRAIT
7½ DAYS
4 DAYS
ICELAND
3½ DAYS
NORTH ATLANTIC
NEWFOUNDLAND
8 DAYS
BEAR ISLAND
4 DAYS
BARENTS SEA
6 DAYS
5½ DAYS
LOFOTEN ISLANDS
3½ DAYS
NORTH CAPE
WHITE SEA
2½ DAYS
FAROES
SHETLANDS
NORTH SEA

Fig. 1. The distant water fishing grounds, with the corresponding journey times.

At each fishing ground 100 cod, between 60 and 70 cm long, were obtained from 3 separate hauls. The fish were eviscerated and washed, and 6 were taken for immediate bacteriological examination. A further 30, required for subsequent examinations after 5, 10, 15 and (in the case of the second expedition) 20 days storage, were placed in ice in 5 metal boxes. Thermocouples were inserted in 2 of the fish in each box and continuous temperature records were kept over the whole period of the experiment.

At each occasion of sampling total colony counts ($/cm^2$) were obtained from a measured area (7·5 cm^2) of skin on each of the six fish. The medium used consisted of: Lab-Lemco, 5 g; peptone (Difco), 10 g; agar (Difco), 15 g; aged sea water, 1 litre. It was adjusted to pH 7·4 and sterilized at 15 lb/in^2 for 15 min. The Astell roll tube technique was employed and the colonies counted after incubation at 20° for 5 days.

For the analysis of populations, colonies were selected from the roll tubes. Each tube was marked off into numbered squares, all the colonies were taken from random squares chosen by reference to tables of random numbers, and plated on sea water agar to obtain pure cultures.

From each sample of fish 100 pure cultures were isolated and their identities determined by means of the screening technique outlined in Fig. 2. The tests used and the taxonomic positions of the various groups have already been fully discussed in our previous paper in this Symposium (Shewan, Hobbs & Hodgkiss, 1960).

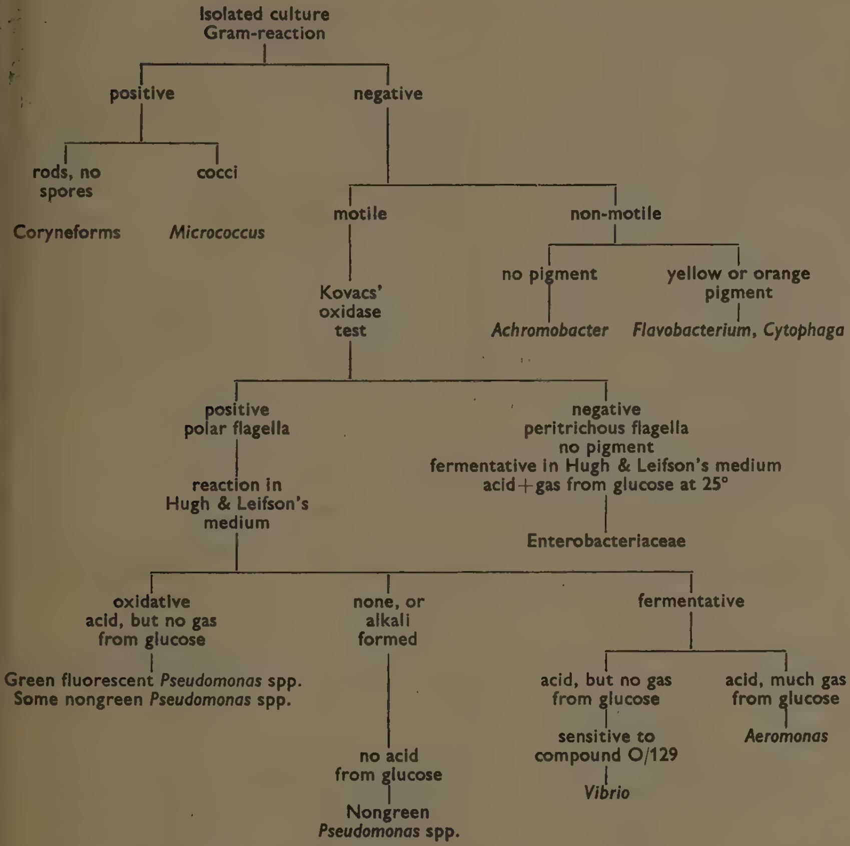
Isolated culture
Gram-reaction
positive
negative
rods, no spores
cocci
Coryneforms
Micrococcus
motile
non-motile
no pigment
yellow or orange pigment
Achromobacter
Flavobacterium, *Cytophaga*
Kovacs' oxidase test
positive
polar flagella
negative
peritrichous flagella
no pigment
fermentative in Hugh & Leifson's medium
acid + gas from glucose at 25°
Enterobacteriaceae
reaction in Hugh & Leifson's medium
oxidative
acid, but no gas from glucose
Green fluorescent *Pseudomonas* spp.
Some nongreen *Pseudomonas* spp.
none, or alkali formed
no acid from glucose
Nongreen *Pseudomonas* spp.
fermentative
acid, but no gas from glucose
sensitive to compound O/129
Vibrio
acid, much gas from glucose
Aeromonas

Fig. 2. An outline of the sequence of tests used in the screening of cultures.

Results and Discussion

The results obtained on the two expeditions are summarized in Tables 1 and 2. The numbers in the individual groups are estimates calculated from the total colony counts by use of the percentage values obtained after the identification of 100 isolates at each time of examination. It must be stressed that the temperature records showed that throughout the experiments all the fish were held within the range 0–1·5°.

The results obtained for freshly caught North Sea and Faroes cod are similar to those previously recorded by Georgala (1957) for North Sea cod, the outstanding feature being the predominance of the *Pseudomonas–Achromobacter* group: but on the North Cape cod the proportion of this group was somewhat lower and the proportion of coryneforms was higher. However, comparison of the results from the three fishing grounds shows that the differences in bacterial flora are small and barely significant statistically.

The important feature of these results, and of those of other workers, is the increase during stowage in ice in the *Pseudomonas–Achromobacter* group at the expense of the rest. This kind of result has led to the general belief that *Pseudomonas* strains are probably the most important of the organisms concerned in the spoilage of fish. Castell and his co-workers (Castell & Anderson, 1948; Castell, Greenhough & Jenkins, 1957; Castell & Greenhough, 1957; Castell, Greenhough & Dale, 1959) have shown that some of the spoilage odours occurring in spoiling fillets can be reproduced by inoculating sterile pieces of fish with pure cultures of *Pseudomonas* strains, and in a few similar experiments carried out at the Torry Research Station it was found that strains of *Pseudomonas* produced fairly rapid breakdown of the fish muscle and typical spoilage odours. Strains of *Achromobacter*, on the other hand, produced little visible change in the muscle and only slight 'off' odours. Work along these lines is being continued.

Table 1. *Changes in the proportions and numbers of different bacteria on the skin of fish stowed at* 0–1·5° *for various periods during expedition* 1, *June* 1959

Fishing ground	Genus and/or kind of bacteria	Proportions (P)* and calculated nos. (N)† of various bacteria on fish stowed at 0–1·5° for (days)							
		0		5		10		15	
		P*	N†	P	N	P	N	P	N
North Cape	*Pseudomonas* nonfluorescent	12	15·79	6	9·63	45	299·25	62	12,809
	fluorescent	2	2·64	11	17·65	5	33·25	20	4,132
	Achromobacter	33	43·40	49	78·65	38	252·50	14	2,893
	Flavobacterium	4	5·27	0	0·00	0	0·00	2	413
	Coryneforms	41	53·90	33	52·97	12	80·00	2	413
	Micrococcus	8	10·50	1	1·60	0	0·00	0	0
	Unidentified	0	0·00	0	0·00	0	0·00	0	0
	Total colony count‡		131·50		160·50		665·00		20·660
Faroes	*Pseudomonas*								
	nonfluorescent	11	2·26	66	315·48	45	1,269·0	79	47,005
	fluorescent	0	0·00	18	86·04	5	141·0	11	6,545
	Achromobacter	59	12·10	13	62·14	31	874·2	0	0
	Flavobacterium	1	0·20	0	0·00	3	84·6	1	595
	Coryneforms	20	4·10	2	9·56	9	253·8	5	2,975
	Micrococcus	9	1·84	1	4·78	7	197·4	2	1,190
	Unidentified	0	0·00	0	0·00	0	0·0	2	1,190
	Total colony count‡		20·50		478·00		2,820·0		59,500
North Sea	*Pseudomonas*								
	nonfluorescent	26	24·49	18	88·74	71	9,372·0	71	39,660
	fluorescent	0	0·00	15	73·95	13	1,716·0	11	6,145
	Achromobacter	33	31·09	26	128·18	7	924·0	13	7,262
	Flavobacterium	0	0·00	8	39·44	0	0·0	0	0
	Coryneforms	25	23·55	12	59·16	8	1,056·0	3	1,676
	Micrococcus	14	13·19	21	103·53	0	0·0	0	0
	Unidentified	2	1·88	0	0·00	1	132·0	2	1,117
	Total colony count‡		94·20		493·00		13,200·0		55,860

* Percentages, based on 100 isolates at each examination. † Numbers (10^3/cm² of skin), calculated from the percentages and the total colony counts/cm² of skin. ‡ Total colony counts, observed values, in 10^3/cm² of skin.

Table 2. *Changes in the proportions and numbers of different bacteria on the skin of fish stowed at* 0–1·5° *for various periods during expedition* 2, *September* 1959

Fishing ground	Genus and/or kind of bacteria	Proportions (P)* and calculated nos. (N)† of various bacteria on fish stowed at 0–1·5° for (days)									
		0		5		10		15		20	
		P*	N†	P	N	P	N	P	N	P	N
North Cape	*Pseudomonas*										
	nonfluorescent	35	4·48	50	67·50	31	1,237	32	517	45	73,350
	fluorescent	0	0·00	1	1·35	0	0	1	16	6	9,780
	Achromobacter	24	3·07	41	55·35	46	1,835	38	614	9	14,670
	Flavobacterium	1	0·13	2	2·70	0	0	14	226	20	32,600
	Coryneforms	22	2·82	5	6·75	18	718	8	129	14	22,820
	Micrococcus	18	2·30	1	1·35	5	200	7	113	5	8,150
	Unidentified	0	0·00	0	0·00	0	0	0	0	1	1,630
	Total colony count‡		12·80		135·00		3,990		1,615		163,000
Faroes	*Pseudomonas*										
	nonfluorescent	26	12·70	23	9·24	20	391	42	6,058	38	9,521
	fluorescent	0	0·00	24	9·65	4	78	3	433	10	2,506
	Achromobacter	19	9·27	26	10·45	26	508	13	1,875	10	2,506
	Flavobacterium	4	1·95	1	0·40	0	0	26	3,750	5	1,253
	Coryneforms	48	23·42	17	6·82	25	489	7	1,009	23	5,763
	Micrococcus	3	1·46	8	3·22	25	489	8	1,154	10	2,506
	Unidentified	0	0·00	1	0·40	0	0	1	144	4	1,000
	Total colony count‡		48·80		40·18		1,955		14,423		25,055
North Sea	*Pseudomonas*										
	nonfluorescent	30	8·94	39	20·98	48	4,986	59	33,850	68	238,816
	fluorescent	0	0·00	11	5·92	16	1,664	21	12,030	9	31,608
	Achromobacter	32	9·54	24	12·91	6	630	5	2,867	15	52,680
	Flavobacterium	10	2·98	2	1·07	1	104	5	2,867	4	14,048
	Coryneforms	25	7·45	23	11·84	16	1,664	9	5,162	3	10,536
	Micrococcus	0	0·00	1	0·54	11	1,144	0	0	0	0
	Unidentified	3	0·89	1	0·54	2	208	1	574	1	3,512
	Total colony count‡		29·80		53·80		10,400		57,350		351,200

* Percentages, based on 100 isolates at each examination. † Numbers ($10^3/\text{cm}^2$ of skin), calculated from the percentages and the total colony counts/cm² of skin. ‡ Total colony counts, observed values, in $10^3/\text{cm}^2$ of skin.

From the kind of evidence discussed it seems apparent that in the spoilage of white fish the characteristic odours and flavours are probably a direct result of the action of nonpigmented *Pseudomonas* strains on the fish muscle. This is borne out by the reactions of these strains in laboratory cultures, where they produce powerful proteolytic enzymes which attack a wide range of proteins.

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(Symposium on *Pseudomonas* and *Achromobacter*: Contribution relating to Paper V)

THE BACTERIAL FLORA OF FISH CAUGHT IN THE PACIFIC

BY J. LISTON

College of Fisheries, University of Washington, Seattle, 5,
Washington, U.S.A.

IN RELATION to the interesting results just presented (Shewan, Hobbs & Hodgkiss, 1960) concerning the bacterial flora of fish caught in European waters, the following summary of data on the bacterial flora of fish obtained in the Pacific Ocean may be of value for comparison. Table 1 shows some of the findings obtained by my associate Dr. R. R. Colwell and myself from marine animals caught in three areas with widely different temperature and hydrographic conditions. These results are remarkably similar to those for North Sea and Arctic fish and emphasize the notable consistency of the bacterial populations from marine creatures taken in different areas. Gram-negative rods of the type under discussion at this Symposium were predominant in all cases, though there were minor differences in the incidence of particular generic groups in the three areas. Such differences may indeed appear from time to time in the same area.

Table 1. *The distribution of different genera of bacteria on fish from three areas of the Pacific Ocean*

Origin of fish	Initial incub. temp. (°C)	Incidence (%) of the genera					
		Pseudomonas, Vibrio	*Achromobacter*	*Flavobacterium*	*Cytophaga*	*Corynebacterium*	Others
Puget Sound	20	66·3	6·5	10·9	2·2	5·4	8·7
	0	59·8	21·7	0·0	0·0	2·2	16·3
Washington coast	20	34·6	25·0	7·7	7·7	5·8	19·2
South Pacific	20	57·5	17·0	2·1	0·0	2·1	21·3

Table 2 indicates the variation in the biochemical capabilities of the bacterial floras of different marine animals sampled in the same area at the same time. The number of isolates tested was in most cases too small to allow of more than extremely tentative conclusions. However, it appears that the overall biochemical capability might differ more from species to species than the generic composition of the associated bacterial populations. This might have importance in spoilage. In the particular case of the samples from the Pacific salmon (*Oncorhyncus*), sufficient cultures

Table 2. *The biochemical properties of bacteria from marine animals of Gig Harbour, Washington*

Animal sampled	Percentage of isolates with specified reaction						
	Pigment producing *Pseudomonas*	Gelatin lique-fied	Litmus milk pept.	Nitrate reduced	Fermentative ability (glucose)	TMO* reduced	Number of isolates
Oncorhynchus sp.	38·8	68·2	67·1	35·5	10·5	20·5	85
Clupea sp.	13·3	33·3	33·3	0·0	0·0	0·0	15
Loligo sp.	88·8	66·6	77·7	100·0	11·1	0·0	9
Aurelia sp.	30·0	40·0	40·0	50·0	0·0	0·0	10
Total Gig Harbour Sample	46·2	61·3	61·3	36·9	8·4	14·3	119

* TMO, Trimethylamine oxide reduced to trimethylamine (Wood & Baird, 1943).

have perhaps been tested to indicate that the biochemical capabilities of the microflora of this fish do not differ very greatly from those of the microfloras of the gadoid fishes (cod and haddock) studied in Dr. Shewan's laboratory.

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper VI)

THE RELATIONSHIP OF ORGANISMS OF THE GENUS *PSEUDOMONAS* TO THE SPOILAGE OF MEAT, POULTRY AND EGGS

By J. C. AYRES

Dairy and Food Industry Department,
Iowa State University, Ames, Iowa, U.S.A.

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1. Introduction

The micro-organisms that ultimately bring about the spoilage of flesh foods are either present at the time of slaughter, or introduced by workmen and their cutting tools, or by water and air in the dressing, cooling and cutting rooms. Studies of the taxonomic distribution of these contaminants reveal that representatives of any of a number of genera are likely to be recovered from the surfaces of meats (Table 1).

While enormous and varied microbial populations are associated with feathers, hair, skins or hides, feet or hoofs, with the gut, and with excretions voided by animals, the species responsible for the spoilage of refrigerated meats seem to be quite restricted.

2. Numbers of Organisms Associated With Spoilage

After processing, more than 80% of the total population of freshly ground beef (Fig. 1) or 75–80% of the colonies present on chicken parts (Fig. 2) (Ayres, 1951, 1959) may be comprised of chromogenic bacteria, moulds, yeasts and sporeforming

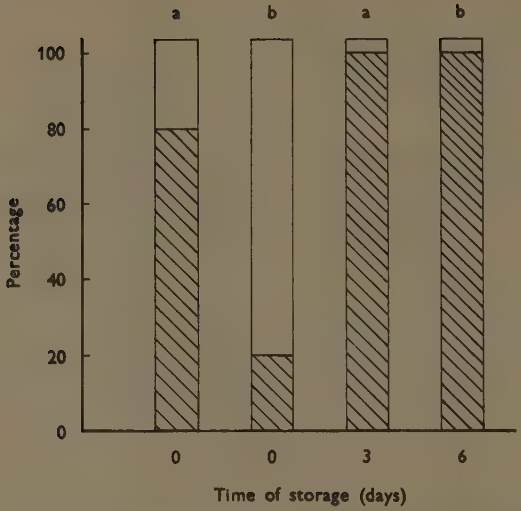

Fig. 1. The percentage distribution by type of organisms from frozen (a) and fresh (b) ground beef at different times during storage at 5°. Hatched areas, typical slime organisms; white areas, miscellaneous.

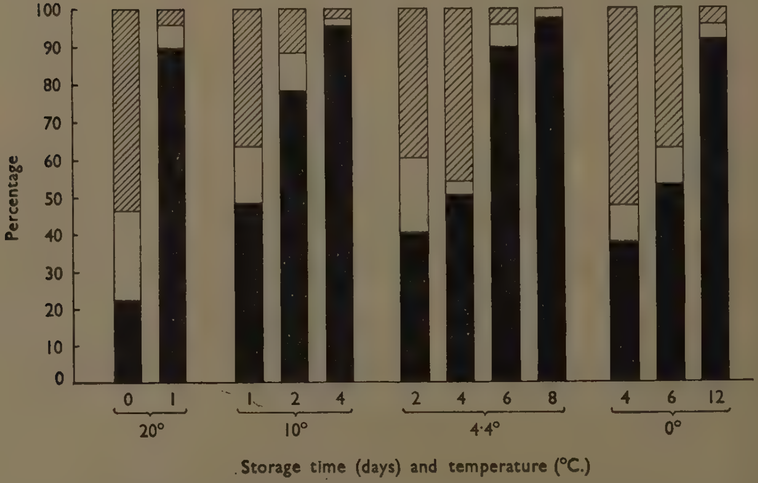

Fig. 2. The effect of storage time at 0°, 4·4°, 10° and 20° on the percentages of various organisms associated with dressed poultry.
Black areas, typical spoilage organisms; hatched areas, chromogens; white areas, miscellaneous.

micro-organisms. However, at the time of spoilage (i.e. the development of off odour and/or sliminess), examination of these meats reveals primarily single, paired, or short chained motile Gram-negative nonsporeforming rods, some of which produce fluorescent yellow, green or bluish pigments: but most do not.

Table 1. *The genera represented among bacteria isolated from the surfaces of meats*

Genus	Genera represented in													
	Fresh beef					Sausage*		Fish†			Chicken			
	1§	2	3	4	5	6	7	8	9	10	11	12	13	14
Pseudomonas	+	+	+		+	+	+	+	+	+		+	+	+
Xanthomonas						+								
Aeromonas					+									+
Azotobacter type		+												
Chromobacterium	+						+							
Alcaligenes					+	+		+				+	+	+(?)
Achromobacter	+	+	+	+	+	+	+	+	+	+	+	+	+	+(?)
Flavobacterium	+			+	+	+	+	+	+	+	+	+	+	
Escherichia					+	+						+	+	
Aerobacter					+	+			+			+	+	
Paracolobactrum					+	+						+	+	
Serratia					+		+		+					
Proteus		+	+		+	+	+	+		+		+	+	
Salmonella					+							+	+	
Haemophilus												+		
Micrococcus	+	+	+	+	+	+	+	+	+	+	+	+	+	
Staphylococcus					+							+	+	
Gaffkya					+							+		
Sarcina			+	+	+	+	+					+	+	
Neisseria						+						+		
Diplococcus				+										
Streptococcus		+											+	
Lactobacillus			+				+							
Corynebacterium												+		
Microbacterium					+	+		+				+		
Bacillus				+	+	+	+					+	+	
Clostridium					+									
Actinomyces		+										+		
Streptomyces					+							+	+	
Bacterium‡				+			+							

* Fresh pork sausage. † 8, cod; 9, haddock; 10, herring.

§ 1, Empey & Scott (1939). 2, Haines (1933). 3, Kirsch *et al.* (1952). 4, Mallmann *et al.* (1940). 5, Ayres (1960). 6, Sulzbacher & McLean (1951). 7, Jensen (1945). 8, Dyer (1947). 9, Stewart (1932). 10, Aschehoug & Vesterhus (1947). 11, Lochhead & Landerkin (1935). 12, Gunderson *et al.* (1947). 13, Ayres *et al.* (1950). 14, Nagel *et al.* (1960).

‡ Genus not recognized in the 7th edition of Bergey's *Manual*.

Several explanations have been offered for the surprising homogeneity in the microflora that persists on raw flesh foods. For example, Haines (1933) reported that the layer of fat and connective tissue covering the surface of the whole beef carcase provides only a poor source of nutrients for many organisms. Also, much of the flora coming into contact with meat consists of mesophiles which grow poorly at low temperatures. If, after killing, the animal is quickly chilled and properly refrigerated, most of these bacteria die. Since organisms that proliferate at low temperatures are not commonly associated with a putrefactive type of decomposition, incipient spoilage of refrigerated meat is ordinarily detected by the development of off odour and slime. Almost 60 years ago, Glage (1901) found the slime that developed on the surface of meat stored at low temperatures and high humidity to be caused by motile aerobic rod-shaped organisms that liquefied gelatin slowly and turned litmus milk alkaline.

Those organisms that find the environment satisfactory multiply abundantly. Various workers refer to certain minimal concentrations of bacteria at the time that incipient spoilage becomes apparent. Schmid (1931) associated the limit of saleability of beef with a bacterial count of 5×10^7–10^8/cm^2 of surface. Haines & Smith (1933) and Empey & Vickery (1933) reported that when bacterial growth is in the advanced stage, meat is slimy to the touch. They respectively considered that this point was attained when the surface loads were $10^{7\cdot5}$ and 5×10^7/cm^2. Lochhead & Landerkin (1935) observed odour from New York dressed poultry when the counts were in the range $2\cdot5 \times 10^6$–10^8/cm^2. The minimal number of organisms required for sliminess of beef was reported by Moran (1935) to be 3×10^6. Ayres (1959, 1960) ascribed off odour in eviscerated poultry and packaged beef to microbial populations in excess of 10^7 cells/cm^2. These meats did not become slimy until loads greater than 5–6×10^7 organisms/cm^2 were recovered (Fig. 3).

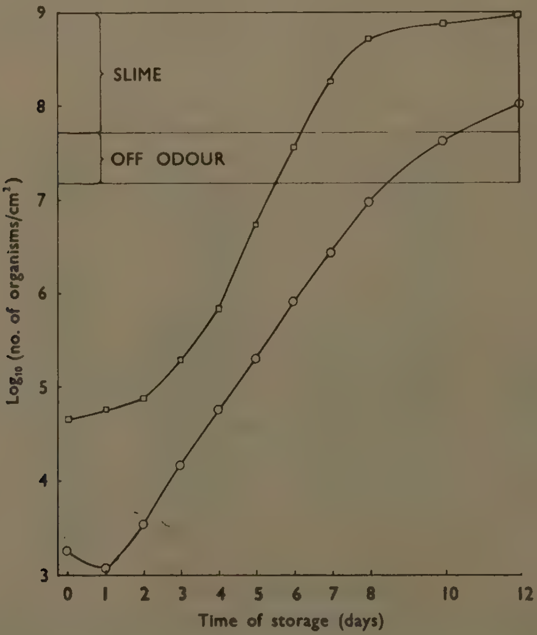

Fig. 3. The development of off-odour and slime on dressed chicken (squares) and packaged beef (circles) during storage at 5°.

3. IMPORTANCE OF PSEUDOMONADS IN MEATS

Haines (1933) stated that ". . . with the exception of a certain number of organisms of the *Pseudomonas* group and a few *Proteus*, the bacteria growing on lean meats stored in the range 4–0°C almost all belong to the *Achromobacter* group". At the same time, but independently, Empey & Vickery (1933) observed that 95% of the initial flora of beef capable of growth at –1° consisted of members of the genus *Achromobacter*. The remainder were species of *Pseudomonas* and *Micrococcus*. During storage, they observed the relative numbers of *Achromobacter* and *Pseudomonas* to increase while those of *Micrococcus* decreased. Empey & Scott (1939) considered that the four principal genera of low temperature bacteria isolated from fresh meat and comprising the flora of the carcase were: *Achromobacter*, 90%; *Micrococcus*, 7%; *Flavobacterium*, 3% and *Pseudomonas*, less than 1%. Jepsen (1947) found *Achromobacter* to be predominant in the slime layer of refrigerated meats. Further, he indicated that 100% of the examined strains showed growth at 2° and that the ability to grow at low temperatures was restricted to the genera *Achromobacter* and *Pseudomonas*. However, Ingram (1934) observed that when varying concentrations of salt were added to small amounts of minced fresh pork and the mixtures stored in Petri dishes in the refrigerator, the surface of the meat was ". . . entirely covered by a fluid film containing a *Pseudomonas* of practically pure culture". This condition prevailed where the concentration of salt was less than 4%. At salt levels ranging between 4 and 10%, moulds predominated.

More recent reports (Jensen, 1944; Ayres, 1951; Kirsch *et al.* 1952; Wolin *et al.* 1957) indicate that species of *Pseudomonas* have a relatively greater importance in causing off odour and slime of flesh foods than was assigned to them by the earlier investigators. It should be remembered that in many of the investigations reported prior to publication of the 5th edition of Bergey's *Manual of Determinative Bacteriology* (1939), only those organisms which formed greenish fluorescent pigment soluble in water were considered to be pseudomonads. Colourless colonies having similar properties were classified in the genus *Achromobacter*. As Brown & Weidemann (1958) so effectively demonstrated in re-assessing the taxonomy of the meat-spoilage bacteria isolated by Empey & Scott (1939) a number of years earlier, classification as pseudomonads on the basis of pigment production is inadequate. In a detailed analysis of the flora on stored poultry, Barnes & Shrimpton (1959) reported that ". . . the predominant spoilage organisms were nonpigmented and pigmented pseudomonads, the former considerably outnumbering the latter. . . ." It was their opinion that growth of nonpigmented pseudomonads explained the poor correlation between development of off odours and appearance of fluorescence.

Attempts by Ayres, Ogilvy & Stewart (1950) to relate the biochemical patterns of bacterial isolates from slimy chicken to those of species identified in the 6th edition of Bergey's *Manual* (1948), revealed certain differences from previously named species (Table 2). Also in 1950, Sulzbacher isolated 28 *Pseudomonas* and 4 *Achromobacter* strains from frozen pork and 6 *Pseudomonas* strains from frozen lamb, all of which showed microscopic growth on agar slants at –4° and grew rapidly at 2°. Most of these organisms were considered to belong to the *Pseudomonas* group but did not fit any of the recognized species. They produced tan- to brown- rather than greenish-pigmented colonies. The organisms formed acid from glucose and galactose and a few other sugars, and produced an alkaline reduction of milk: but they did not reduce nitrates to nitrites. They were considered somewhat similar to *Pseudomonas geniculata*, but the description of that organism was not considered sufficiently complete for certain identification.

Kirsch *et al.* (1952) described the main characteristics of a number of organisms that they had isolated from two samples of refrigerated hamburger. While they indicated that it was not possible to identify the organisms positively with species described in the 6th edition of Bergey's *Manual* (1948), the isolates were grouped on the basis of tests made and where a group differed only slightly from a described species, the probable species name was indicated. Nine of the isolates produced green pigment and were characterized as being similar to *Ps. aeruginosa* but the great majority (532) were motile, nonpigmented pseudomonads. Of these, 147 were considered related to *Ps. geniculata* and 88 to *Ps. fragi*, and a further 62 differed from *Ps. geniculata* only by their failure to liquefy gelatin and were tentatively classified as *Ps. rugosa*; but the rest (235) were not identified as to species.

Table 2. *The relationship of organisms isolated from slimy chickens to described species of* Pseudomonas

(After Ayres, Ogilvy & Stewart, 1950)

Strain no.	Species most closely related according Bergey's *Manual* 6th ed. (1948)	Differences shown by descriptions in the *Manual*	Suggested classification
7–1	*Ps. geniculata*	Sugar reactions not reported	*Ps. fluorescens*
7–2	*Ps. mephitica*	Reduction of litmus milk; maltose acid; slow gelatin liquefaction reported. Growth at 37°	
	Ps. putrefaciens	Rapid reduction of litmus reported; sugar reactions not reported	
7–13	*Ps. fragi* *Ps. multistriata*	Alkali in maltose, and fructose not reported. Gas from glucose reported	*Ps. fragi*
8F–4	*Achromobacter pellucidum* (Bergey's *Manual*, 5th ed., 1939)	Reported no action for dextrose, other sugars not reported. Bergey 6th ed. indicates this organism probably a *Pseudomonas*	
7–19	*Ps. rathonis*	Maltose and fructose not reported	*Ps. putida*
9–16	*Ps. desmolyticum* *Ps. pictorum*	Sugars not reported Fructose not reported	
7–5	*Ps. sinuosa*	Milk reported unchanged	*Ps. ambigua*
6–8	*Ps. ochracea*	Indole, H_2S, glucose not reported	*Xanthomonas* sp. (addendum)
7–7	*Ps. segnis*	Sugars not reported; indole not formed	

In 1954, Ingram & Hobbs reported that *Ps. fluorescens* occurred in hams both before and after curing. Kitchell & Ingram (1956) identified an organism which resembled *Ps. multistriata* as the dominant species in the sliming of pork at 15°.

Wolin, Evans & Niven (1957) also studied the bacterial flora of refrigerated fresh beef and reported that 90% of the isolates were either *Ps. geniculata* or differed from it primarily by an inability to liquefy gelatin. Among the miscellaneous forms, one was identified as *Ps. fragi*, while two strains produced pyoverdine and grew at 37°. Since one of these liquefied gelatin while the other did not, they were considered similar to *Ps. jaegeri* and *Ps. convexa* respectively. Recently Ayres (1960) compared the reactions of pseudomonads isolated from refrigerated sliced beef with those of identified species within the genus. Sixteen different biochemical patterns were observed and the reactions compared with those given for named species in the 7th edition (1957) of Bergey's *Manual* (Table 3).

Table 3. *The relationship of organisms isolated from refrigerated sliced beef to described species of* Pseudomonas

(After Ayres, 1960)

Strain no.	Species most closely related according to Bergey's *Manual* 7th ed. (1957)	Differences shown by descriptions in the *Manual*	Suggested classification
P-92	*Ps. fluorescens*		*Ps. fluorescens*
P-65	*Ps. geniculata*	Sugars not reported	
P-58	*Ps. mephitica*	Skunk-like odour in 1–2 days, and slow gelatin liquefaction reported	
P-7	*Ps. putrefaciens*	Rapid reduction of litmus reported	
P-80	*Ps. syncyanea*		*Ps. fragi*
P-37	*Ps. convexa*		*Ps. convexa*
P-16	*Ps. taetrolens* or *Ps. rugosa*	Some growth at 37° Indole not formed; has musty odour	*Ps. taetrolens* or *Ps. incognita*
P-43	*Ps. putida*	Maltose and fructose not reported	*Ps. putida*
P-13	*Ps. rathonis*		
P-51	*Ps. striata*		
P-26	*Pseudomonas* sp.	Similar to *Ps. sinuosa* reported in 6th ed. but not given in 7th ed.	*Ps. ambigua*
P-34	*Pseudomonas* sp.	Gelatin liquefied; acid and gas from glucose; litmus milk coagulated	*Aeromonas* sp.
A-76	*Ps. ochracea*	Grows well at 25°	*Xanthomonas* sp. (addendum)
A-90	*Ps. segnis*		

4. PSEUDOMONADS ASSOCIATED WITH SPOILAGE OF EGGS

The importance of pseudomonads in the spoilage of eggs is likewise well documented. Early studies by Artault (1894) and Poppe (1910) implicated *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*) and *B. putidus nonliquefaciens* (*Ps. putida*) respectively among organisms that were responsible for egg rot. In 1927 Turner isolated an organism that produced a peculiar penetrating musty odour when introduced into fresh eggs and so suggested the name *Achromobacter perolens*. This bacterium produced optimal growth at room temperature. It was motile by means of a polar flagellum, was capable of penetrating the shell of the egg and produced opalescence in the egg white into which it was introduced. Spanswick (1930) confirmed Turner's findings. Levine & Anderson (1930, 1932) isolated 25 odour-producing organisms from 4 musty eggs. Two types liquefied gelatin and reduced nitrates, as did Turner's organism, but a third did not, and to this they gave the name *Pseudomonas graveolens*. The names *Ps. mucidolens* and *Ps. mucidolens* var. *tarda* were suggested for the other two.

Grabiec, Szybalski & Tuszynski (1949) considered that an organism which they called *Pseudomonas perolens* var. *Gdansk* was responsible for producing a penetrating musty odour in eggs. A year later Szybalski (1950) tested all the organisms previously implicated in the production of musty odour and concluded that *Ps. mucidolens* had lost that property. In addition to producing a green fluorescent water soluble pigment, Szybalski reported that the *Achromobacter perolens* of Turner possessed a single polar flagellum and therefore belonged in the genus *Pseudomonas*.

Richard & Mohler (1950) reported that *Pseudomonas putida*, *Ps. ovalis*, *Ps. viscosa* and *Ps. schuylkilliensis* were able to cause mustiness in eggs. Brooks & Hale (1954) were able to confirm this observation insofar as *Ps. putida* was concerned but, in their experiments, *Ps. schuylkilliensis* did not cause mustiness. Instead, they associated growth of the last named organism with the production of a slightly feculent, 'cabbage-water' smell, a defect earlier ascribed by Haines (1938) to the growth of pseudomonads.

Pavarino (1929) described spoiled eggs in which the yolk was liquefied and the white was watery, turbid and coloured green. *B. fluorescens liquefaciens* (*Pseudomonas fluorescens*), *Micrococcus roseus*, and *Staphylococcus aureus* were isolated. Haines (1938) found 38% of the microflora in washings from shell eggs to consist of nonsporing rods, sporing rods and cocci; *Achromobacter* comprised 19%, *Pseudomonas* 6%, and *Alcaligenes* 4% of the total flora. He attributed green and red or pink rots to strains of *Pseudomonas* and stated further that one type of black rot was caused by certain species of the genus. Haines also demonstrated that *Ps. aeruginosa* was able to produce green rot of shell eggs. Alford *et al.* (1950) reached similar conclusions and, in addition, indicated that rots lacking any characteristic colour may be caused by strains of *Pseudomonas* or *Achromobacter* and that pink rots may be caused by strains of *Pseudomonas*. The Australian investigators concluded that pink rot may merely be a later stage of the type of spoilage that results in green rotting.

Wrinkle, Weiser & Winter (1950) sampled whole egg before and after freezing and concluded that, among the 13 genera isolated most frequently from unpasteurized egg, *Alcaligenes*, *Flavobacterium*, *Proteus* and *Pseudomonas* predominated. From defrosted egg samples they obtained a slightly different flora. Unfortunately in their studies, frozen eggs were held at room temperature during the observation period. They stated that spoilage usually took place less than 24 hr after the samples had completely defrosted.

According to Lorenz *et al.* (1952) the most common type of bacterial spoilage in cold stored eggs is caused by members of the genus *Pseudomonas* and this was frequently the only type found immediately after eggs were removed from the warehouse. Pseudomonad incidence may vary up to 80% or more in individual cases of 30 dozen eggs and entire seasons of cold storage operations have been conducted at a loss due to the high incidence of this type of spoilage.

Florian & Trussell (1957) studied 228 isolates from 81 infected eggs. Of the 197 organisms classified, 72 were found to belong to the genus *Alcaligenes* and 52 to the genus *Pseudomonas*. Considerable variation in the ability of these organisms to infect eggs was demonstrated and these workers considered that the principal spoilage species were *Ps. fluorescens* and *Alc. bookeri* (strain A), which were found in about 50% and 25% of the infected eggs respectively. Of 19 species that they identified only 10 were capable of penetrating the inner membrane of the egg and infecting the egg content. Among these were *Ps. fluorescens* and *Ps. putrefaciens*. Recently Garibaldi (1960) suggested that the conalbumin of egg white may be the antigrowth factor which limits the infection of shell eggs.

The term 'pyoverdine' was suggested by Turfeijer (1941) for the fluorescent water soluble pigment complex produced by pseudomonads. Later, Elliott (1958*a*) reported pyoverdine to be a reversible pH indicator produced more rapidly in aerated media than in substrates having a low oxygen tension, and decomposed by long wave ultraviolet, but not by visible, light. While fluorescence of the pigment in egg was proportional to oxygen tension, its production ceased at an O-R potential below –0·3 V. Elliott used one strain each of *Ps. fluorescens* and *Ps. ovalis* and found that the *Ps. fluorescens* produced less pyoverdine in eggs and cultures than did the *Ps. ovalis*. He observed (1958*b*) that pseudomonads form pyoverdine readily in shell egg spoilage, but much less readily in liquid egg spoilage. In addition to the species already listed, Elliott (1960, pers. comm.) isolated *Ps. geniculata* and *Ps. convexa* from shell eggs.

5. Differentiation of Genera Recovered from Meat or Egg Products

Although the pseudomonads are much more prevalent than other micro-organisms on refrigerated meat or egg products, isolates cannot be classified as to genus until a few selected biochemical reactions have been determined and the organisms have been examined microscopically to ensure that they are Gram-negative rods with polar flagella, as shown in Table 4. In addition to the identifying differences listed there, each of the genera listed can be further differentiated on the basis of other characteristics. Two other genera with which pseudomonads may sometimes be confused, *Vibrio* and *Chromobacterium*, are not included in the key. Difficulty in determining the borderline between straight rods and curved rods or in identifying the pigment formed often leads to the incorrect assignment of bacteria to the genus *Pseudomonas*, and vice versa. Shewan *et al.* (1954), Sneath (1956) and Rhodes (1957) have described methods whereby sharper differentiation between pseudomonads and other genera may be effected.

Table 4. *Key for the identification of Gram-negative rod-shaped organisms isolated from meat and egg products*

(Data from Bergey's *Manual*, 7th ed., 1957)

Gram-negative, nonsporeforming rods.	
Motile by means of peritrichous flagella or nonmotile.	
Glucose attacked oxidatively if at all.	
Chromogenic.	
Pigment not water soluble.	*Flavobacterium*
Non-chromogenic.	
No acids from hexoses; litmus milk alkaline.	*Alcaligenes*
Acids from hexoses; litmus milk unchanged or slightly acid.	*Achromobacter*
Motile by means of polar flagella.	
Glucose attacked fermentatively.	
Alcohol produced.	*Zymomonas*
Acid or acid and gas produced.	
Fix free atmospheric nitrogen.	*Azotomonas*
Do not fix free atmospheric nitrogen.	
Luminescent in media containing 5% salt.	*Photobacterium*
Non-luminescent; produce 2,3-butanediol.	*Aeromonas*
Glucose attacked oxidatively if at all.	
Produce acetic acid from ethanol.	*Acetobacter*
Do not produce acetic acid.	
Produce yellow pigment, not water soluble.	*Xanthomonas*
Pigment, when produced, water soluble.	*Pseudomonas**

* Other bacteria having polar flagella that may or may not attack glucose include species belonging to the genera *Chromobacterium* and *Vibrio*. These organisms may be distinguished from pseudomonads by the following:

Chromogenic; violet pigment insoluble in water; H_2O_2 sensitive (Sneath, 1956).	*Chromobacterium*
Curved rods; sensitive to penicillin and O/129 (Shewan, Hodgkiss & Liston, 1954).	*Vibrio*

6. DIFFICULTIES IN IDENTIFYING SOIL AND FRESH WATER PSEUDOMONADS

Even when the original cultures are available, numerous difficulties are encountered in attempting to identify organisms in accordance with the description given in the 7th edition (1957) of Bergey's *Manual*. Many of the organisms initially isolated from food products, water and other sources are no longer available. In addition, organisms from poultry or meat and characterized as being similar to *Ps. sinuosa*, *Achromobacter pellucidum* and *Ps. multistriata* in accordance with the 5th or 6th editions of Bergey's *Manual* are no longer recognized as species in the 7th edition. It would be impossible to establish the validity of status for the 49 soil and fresh water pseudomonads defined in the 7th edition without thoroughly and exhaustively studying every reaction of each of these bacteria. All too often the reactions of pseudomonads isolated in nature fail to correspond with those of species recognized in the *Manual*. Other biochemical reactions are not sufficiently stable to be dependable. As has been pointed out by other workers (Seleen & Stark, 1943; Rhodes, 1959), the property of producing fluorescent pigment and of musty and/or aromatic odours is unstable and depends upon the substrate. Earlier, Rhodes (1957) pointed out that the ability of cultures to digest milk or gelatin was impaired or lost when cultures were maintained in peptone broth and put forward the hypothesis that such loss of proteolytic ability is explicable on the basis of adaptation to environment. One such factor deserving of consideration is the effect of temperature. Many variations in describing optimal temperatures for growth of the various species of pseudomonads may be found in Bergey's *Manual*. Often the organisms were tested at only one or two incubation temperatures.

It is of interest to this discussion that except for odour, action on three sugars, and reduction of nitrates to nitrites, the descriptions given in Bergey's Manual for *Ps. perolens* and *Ps. fragi* are similar. Tests conducted by the writer indicated that a *Ps. perolens* isolate which rapidly liquefied gelatin and completely coagulated litmus milk at 15° had much less growth or proteolytic activity at 30° and failed to grow at 37°. Also, at the lower temperature it produced odour and sugar reactions similar to those of *Ps. fragi*. While the organism reduced nitrates to nitrites, it did not produce a musty odour in eggs. The effect of temperature on the lipase activity of *Ps. fragi* was previously reported by Nashif & Nelson (1953*a*,*b*). In addition, Greene & Jezeski (1954) noted that a proteolytic strain of *Pseudomonas* which liquefied gelatin at 0, 10 and 20° lost this ability at higher temperatures. Conversely, this pseudomonad was able to ferment glucose at 30° but not below. Recently, Alford (1960) demonstrated that a slight increase in temperature inhibited liquefaction of gelatin, lipolysis, citrate utilization, acid production from xylose and arabinose and action on litmus milk without apparently affecting the growth of several cultures of organisms belonging to the genera *Pseudomonas* and *Achromobacter*. On the basis of studies made at 20, 31–33 and 35–37°, Alford concluded that the greatest effect of temperature on biochemical activity was observed in the range 31–37°. He cautioned that false negative reactions may be encountered if the procedure of incubating at temperatures at which most rapid growth occurs is used.

The classification of soil and fresh water pseudomonads given in Bergey's *Manual* is limited in value since it is based primarily upon unstable properties such as pigment production, gelatin liquefaction, reaction in litmus milk, formation of indole and action on nitrates. While the work reported here substantially agrees with that of Seleen & Stark (1943), Haynes (1951) and Rhodes (1959), the regrouping of these organisms on the basis of information other than that given in the *Manual* must necessarily be halted until a more satisfactory system can be found.

7. ALTERNATIVE CLASSIFICATION SCHEME

The search for an alternative scheme of classification is not an easy assignment. As may be seen in Tables 5 and 6, eleven distinct classification groups can be made on the basis of temperature requirements, gelatin liquefaction, indole formation and nitrate reduction. If indole production from tryptophane is discarded as an unstable property, the 49 species present 8 distinct patterns. These 'species groups' are: *Ps. aeruginosa*, *reptilivora*, *ambigua*, *putida*, *convexa*, *fluorescens*, *fragi* and *taetrolens*. In Rhodes' characterizations (Rhodes, 1959) no differences were reported between *Ps. fluorescens* and the species *schuylkilliensis*, *geniculata*, *septica*, *incognita*, *mildenbergii*, *convexa*, *eisenbergii*, *cohaerens* and *denitrificans*, and only minor differences were observed from the species *syncyanea*, *taetrolens*, *rugosa*, *putrefaciens*, *striata*, *ovalis*, *mephitica*, *mira* and *putida*. If it is assumed that the relationship of these

Table 5. *A suggested grouping of soil and fresh water pseudomonads on the basis of temperature requirements and common biochemical reactions*

Species Bergey's *Manual* 7th ed.	M* or N.D.	Growth at 42°	37°	30°	25°	Gelatin liquefaction	Indole formation	Nitrate reduction	Suggested classification
aeruginosa		+	+			+	−	+	*Ps. aeruginosa*
pseudomallei		+	+			+	−		
boreopolis			+			+		+	
effusa			+			+	−	+NH_3	
ephemerocyanea			+	+	+	+	−	+	
iodinum			+	+	+	+	−	+	
reptilivora			+		+	+	−	−	*Ps. reptilivora*
smaragdina			+			+	−	−	
caviae			+	+	+	+	+	+	*Ps. caviae*
ambigua			+	+	+	−	+		*Ps. ambigua*
sinuosa†			+	+	+	−	+	−	
arvilla			+	+	+	−		−	
dacunhae			+	+	+	−		−	
salopia			+	+	+	−		−	
cruciviae			+	+	+	−		−	
putida	M		+	+	+	−	−	+	*Ps. putida*
oleovorans			+	+	+	−	−	+	
stutzeri			+	+	+	−		+N_2	
tralucida			+	+	+	−	−	+	
lasia			+	+	+	−	−	+	
desmolytica			+	+	+	−		±	
rathonis			+	+	+	−		±	
ovalis	M		+	+	+	−	−		
striata	M		+	+	+	−	−		
convexa	N.D.		−		+	−	−		*Ps. convexa*
eisenbergii	N.D.				+	−	−	+	
riboflavina				+		−		+	
denitrificans	N.D.				+	−	−	+N_2	
indoloxidans					+	−	−	+	
mira	M				+	−	−	+NH_3	
fluorescens					+	+	−	+NH_3	*Ps. fluorescens*
putrefaciens	M		−		+	+	−	+NH_3	
mephitica	M		−	scant	+	+	−	+	
chlororaphis				+	+	+	−	+	
perolens			−	+	+	+	−	+NH_3	
geniculata	N.D.		−		+	+	−		
cohaerens	N.D.				+	+	−		
synxantha			−		+	+	−		
septica	N.D.				+	+			
fragi			−		+	+	−	−	*Ps. fragi*
multistriata†			−		+	+	−	−	
pavonacea			scant		+	+	−	−	
syncyanea	M				+	+	−	−	
erythra				+	+	no growth	−	−	
myxogenes			−	+	+	+	+	+NH_3	*Ps. myxogenes*
schuylkilliensis	N.D.				+	+	+		
fairmontensis			−		+	+	+		
taetrolens	M			+	+	−	−	−	*Ps. taetrolens*
mildenbergii	N.D.				+	−	−	−	
incognita	N.D.		−		+	−	+		*Ps. incognita*
rugosa	M		−	+	+	−	+		

* M or N.D., extent of difference from *Ps. fluorescens* according to Rhodes (1959): M, minor; N.D., no difference. †, 6th edition.

organisms to *Ps. fluorescens* is typical for other members of the series in which they are representative, the only patterns consistent for pseudomonads growing on flesh foods are those for groups to which *Ps. fluorescens* and *Ps. ambigua* belong. In eggs, spoilage resulting from growth of *Ps. aeruginosa* has also been cited.

Table 6. *A key to the species of* Pseudomonas *found in soil and water*

Grows readily at 42°.	*Ps. aeruginosa*
Grows more readily at 37° than at 5°.	
Gelatin liquefied.	
Indole formed; nitrate reduced.	*Ps. caviae*
Indole not formed; nitrate not reduced.	*Ps. reptilivora*
Gelatin not liquefied.	
Indole formed.	
(Indole not given, nitrate not reduced *Ps. sinuosa*)	*Ps. ambigua*
Indole not formed.	
Nitrate reduced.	*Ps. putida*
Grows more readily at 5–30° than at 37°.	
Gelatin liquefied.	
Indole formed.	
Nitrate reduced.	*Ps. myxogenes*
Indole not formed.	
Nitrate reduced.	*Ps. fluorescens*
Nitrate not reduced.	*Ps. fragi*
Gelatin not liquefied.	
Indole formed.	*Ps. incognita*
Indole not formed.	
Nitrate reduced.	*Ps. convexa*
Nitrate not reduced.	*Ps. taetrolens*

Colony characteristics, utilization of carbohydrates, phenol, and naphthalene, hydrolysis of lipids, production of catalase, hydrogen sulphide and/or hydrogen peroxide are also among the properties that have been proposed for assigning species status to members of this group of organisms. However, information concerning reactions of the several species with regard to these properties is fragmentary.

Differences are often detectable between organisms within a species classification in the appearance of their colonies on agar, gelatin or potato slants, and in their pigmentation and production of fluorescence, but the use of these cultural characteristics for species designation is of doubtful value. Rhodes (1959) came to similar conclusions with the 165 isolates she studied and further reported that ". . . different batches of potatoes gave rise to different types of growth and pigmentation".

Data concerning the utilization of carbohydrates by most of the species within this group are inadequate. For *Ps. aeruginosa* and *Ps. fragi* the reactions with 12 sugars or alcohols are reported, and for an additional 17 species the actions on 4 or more carbohydrates are given; but no information is provided concerning sugar reactions for 17 of the remaining 30 organisms and acidity in glucose is the only reported test for 6 others. Action on cellulose is given for 7 of the 49 organisms characterized. Three of the species attack phenol and 5 attack naphthalene, but no information is given concerning the ability of any of the other organisms to utilize these compounds.

Almost no data are provided concerning the hydrolysis of lipids and the production of catalase and/or hydrogen peroxide. Two species, *Ps. pavonacea* and *Ps. taetrolens*, are reported to produce hydrogen sulphide, while 4 others did not display this property.

8. NEED FOR SYSTEMATIC STUDY

A systematic study of species within the genus is urgently needed. It is conceivable that through such an approach pseudomonads may be separated into a few general groups on the basis of distinct and stable characteristics. Before this is possible it will be necessary to have much information that is still lacking. Reaction rates and profuseness of growth at each of several temperatures, such as 5, 15, 25, 37 and 42°, should be determined. Procedures relating to age of culture at time of transfer, size of inoculum and medium used for growth should be standardized. Also, the series of biochemical tests performed by each of the several laboratories investigating these organisms should be selected so that similar carbon, nitrogen, and lipid sources are used for each isolate. Uniformity in the reporting of results is also needed. Completed data sheets similar to the Standard Descriptive Charts issued by the Society of American Bacteriologists (Manual, 1957) should be available for each species of *Pseudomonas* listed in Bergey's *Manual*. If the Chart that is now available is to be adapted for characterizing the pseudomonads, information concerning biochemical reactions should be revised and much of the detail that is requested regarding cultural characteristics should be deleted.

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THE EFFECTS OF ANTIBIOTICS AND IRRADIATION ON THE *PSEUDOMONAS–ACHROMOBACTER* FLORA OF CHILLED POULTRY

BY MARGARET J. THORNLEY, M. INGRAM AND ELLA M. BARNES
Low Temperature Research Station, Cambridge

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1. INTRODUCTION

IT IS NOW well established that eviscerated chickens stored under refrigeration are usually spoiled by organisms resembling *Pseudomonas* and *Achromobacter*. Until recently, however, when new methods were introduced for the identification of these bacteria, little attempt had been made to classify them further or to determine whether changes in the environment led to any alteration in the types present. In this paper the effect of antibiotics and irradiation on the growth of these organisms on poultry is discussed, mainly on the basis of our experiments at the Low Temperature Research Station. These experiments were all done on chilled eviscerated poultry stored at either 1° or 3°. The chickens were generally processed in a very large poultry plant where daily several thousand birds were killed, eviscerated and ice slush cooled (Barnes & Shrimpton, 1958). In two experiments, however, they were processed in a farm scale plant using dry evisceration following overnight chilling in air. The methods of sampling and bacteriological examination have been described previously (Barnes & Shrimpton, 1958).

2. NORMAL SPOILAGE FLORA OF CHILLED POULTRY

When a carcase leaves the poultry plant it carries a diverse microbial flora acquired in the plant during eviscerating and processing. Ayres (1959), in a recent review, stated that chromogenic types accounted for at least half of the total bacterial population of processed poultry; *Pseudomonas* and nonpigmented cocci comprised one fifth to one quarter of the population, the remaining organisms consisting of yeasts and miscellaneous bacteria. Gram-negative cocci, with properties similar to *Alcaligenes*, have been found occasionally to persist during all stages of spoilage (Ayres, Ogilvy & Stewart, 1950), and it is possible that these are similar to the organisms described below as *Achromobacter*.

Changes in the microbial population of chilled eviscerated chickens after storage at 1° were studied by carrying out viable counts on heart infusion agar (Difco) incubated at 1°, 20° and 37°. Faecal streptococci were also determined. These results, similar to those obtained on many occasions, have already been reported by Barnes & Shrimpton (1958) and are summarized in Table 1. Organisms which were able to grow to visible colony size in 14 days at 1° are referred to in this paper as psychrophiles, and it can be seen that such organisms initially formed about one fifth of the total population. However, during storage of the chickens at 1°, the psychrophiles grew and eventually predominated on the spoiled carcases; this pattern was also reported by Ayres (1959). The 37° count at the outset probably indicated faecal organisms, but after storage it represented only the small proportion of psychrophiles which were able to grow overnight at 37°; this count thus lost its significance. Because the psychrophiles were responsible for the spoilage of the poultry, only the organisms growing on the plates at 1° have been isolated and identified; thus, any *Pseudomonas* or *Achromobacter* strains which may have been present on the processed chickens but were unable to multiply rapidly at low temperature are not included in the discussion below.

Table 1. *The relationship between the development of 'off' odours in chickens stored at* 1°, *colony counts at* 1°, 20° *and* 37°, *and numbers of faecal streptococci*

Stage of storage	Colony count/cm²* at			No. of faecal streptococci
	1°	20°	37°	
Initial	$3{\cdot}5 \times 10^4$	$1{\cdot}8 \times 10^5$	$4{\cdot}4 \times 10^4$	$5{\cdot}3 \times 10^3$
Slight 'off' odour	$7{\cdot}5 \times 10^7$	$7{\cdot}4 \times 10^7$	$6{\cdot}2 \times 10^5$	$1{\cdot}7 \times 10^4$
Strong 'off' odour	$7{\cdot}8 \times 10^8$	$1{\cdot}0 \times 10^9$	$1{\cdot}8 \times 10^7$	$5{\cdot}0 \times 10^4$

* Mean of duplicate results obtained from 3 chickens.

As Table 1 shows, the numbers of psychrophilic bacteria increased about a thousandfold from the initial state to the first definite trace of unusual odour, and about a further tenfold by the time the carcase had acquired a strong smell and was frankly spoiled. These large changes in numbers of bacteria must be borne in mind when considering changes in the proportions of different types during the course of spoilage.

(a) *Identification of organisms*

Before analyzing the types of psychrophiles present on the poultry, it is necessary to discuss the differentiation of the strains of *Pseudomonas* and *Achromobacter* isolated. The tests used were mainly those suggested by Dr. J. M. Shewan and are listed in Table 2. They were applied to bacteria already found to be Gram-negative, and served both to separate *Pseudomonas–Achromobacter* from other groups, and to distinguish between pigmented and nonpigmented *Pseudomonas* and *Achromobacter*.

Table 2. *Tests used to differentiate* Pseudomonas, Achromobacter *and other Gram-negative bacteria*

Character	Genus or group		
	Pseudomonas	*Achromobacter*	Coli-aerogenes
Motility	+	+ or −	+ or −
Morphology	Rods	Rods, coccoid rods or cocci, often in pairs	Rods
Flagella	Polar	Peritrichous	Peritrichous
Fluorescence[1]	+ or −*	−	−
Pencillin sensitivity[2]	−	+ (90% of strains)	+ or −
Oxidase[3]	+	+ or −	−
Mode of attack on glucose[4]	Oxidative	Oxidative or inert	Fermentative with gas
Arginine[5]	+	−	+ or −

* Shewan's group 1 pigmented strains, +; group 2 nonpigmented strains, −.

[1] King, Ward & Raney (1954). [2] Shewan, Hodgkiss & Liston (1954). [3] Kovacs (1954). [4] Hugh & Leifson (1953). [5] Thornley (1960).

Fluorescence of a solid streak of growth on the medium of King, Ward & Raney (1954) was always accompanied by green pigment, and indicated pigmented strains of *Pseudomonas*. This property has remained constant for any given culture over a period of several years. All the pigmented strains were motile rods, with polar flagella in the few cases examined, were resistant to 2·5 i.u./ml of penicillin, were oxidase positive, had an oxidative metabolism of glucose, and all except one produced alkaline conditions anaerobically in an arginine medium (Thornley, 1960).

The nonpigmented *Pseudomonas* had the same combination of properties except for the absence of fluorescence and pigment. All strains tested attacked arginine.

The strains of *Achromobacter* were mostly nonmotile square-ended short rods, coccoid rods or occasionally kidney shaped cocci resembling *Neisseria*; they sometimes showed a mixture of Gram-positive and Gram-negative cells on first isolation. The few motile strains were found to be peritrichous. Most of them could be distinguished from *Pseudomonas* by sensitivity to penicillin, but about 10% of our strains were resistant. The oxidase reaction could be either positive or negative, while acid from glucose was produced either oxidatively or not at all. All the strains were arginine negative.

Other than *Pseudomonas–Achromobacter*, the commonest Gram-negative organisms found were coli-aerogenes bacteria and the results for these are shown for comparison. It can be seen that the combination of tests employed was able to distinguish *Pseudomonas–Achromobacter* from coli-aerogenes bacteria and, indeed, from any other Gram-negative organisms tested.

(b) *Distribution of* Pseudomonas–Achromobacter *initially and during storage*

The psychrophilic population, on the processed and stored control chickens from two experiments, was analyzed with the results summarized in Table 3. It is evident that whereas pigmented *Pseudomonas* and *Achromobacter* formed the major portion of the psychrophiles present initially, after storage the nonpigmented *Pseudomonas* predominated. This corresponds broadly to earlier experience with chilled meat (Haines, 1933; Empey & Scott, 1939), though nonpigmented *Pseudomonas* was then called *Achromobacter* (Brown & Weidemann, 1958).

Table 3. *Changes in the distribution of* Pseudomonas *and* Achromobacter *during storage of chickens at* 1°

Stage of storage	No. of strains	Distribution (%) of types from counts at 1°			
		Pseudomonas		*Achromobacter*	Miscellaneous bacteria
		Pigmented	Nonpigmented		
Initial after processing	58	34	11	34	21
Slight 'off' odour	29	17	64	8	11
Strong 'off' odour	62	16	58	14·5	11·5

When the nonpigmented *Pseudomonas* have multiplied to reach a level exceeding $10^7/\text{cm}^2$ on the surface of the carcase a characteristic putrid ammoniacal smell appears which, more than the slimy feeling to the touch, signals that the chicken is beginning to spoil. This, too, has been observed by others with chickens, notably by Ayres (1959), and corresponds also to experience with other meats (Haines, 1933).

The changes which may take place in the normal spoilage pattern of carcases stored at 1° or 3° following treatment with antibiotics or radiation are discussed below.

3. Effect of Antibiotics

Of all the antibiotics so far tested for food preservation the tetracyclines have proved the most useful for extending the storage life of chilled poultry, and in several countries (but not in Great Britain) both chlortetracycline and oxytetracycline are being used for this purpose. In the U.S.A., where almost all the trade is in prepacked eviscerated poultry, 10 p/m of chlortetracycline or oxytetracycline can now be incorporated in the ice slush used for chilling the eviscerated carcases. The regulation permits a tolerance level of 7 p/m, which is not to be exceeded in any part of the carcase.

Early reports suggested that there was no significant change in the types of spoilage organisms present following antibiotic treatment. It was later shown by Ayres *et al.* (1956) that there was a considerably greater number and proportion of yeasts on spoiling antibiotic-treated carcases, a result confirmed by many others including the present authors. Simpson *et al.* (1959), however, still maintain that spoilage of chlortetracycline-treated 'processed fryers' is due to bacterial rather than yeast growth. Little, however, has been published about the effect of the antibiotics on the several elements of the bacterial population itself.

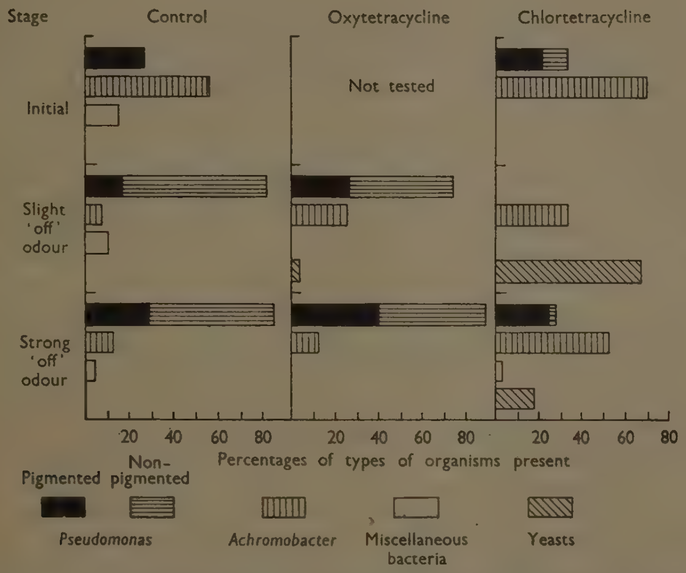

Fig. 1. Changes during storage in the types of psychrophilic spoilage organisms on control and antibiotic treated poultry.

Barnes & Shrimpton (1958) compared the effect of concentrations of 10 p/m of chlortetracycline, oxytetracycline or tetracycline in the ice slush on the storage life and spoilage flora of carcases stored at 1° and the results are summarized in Fig. 1. On both the control chickens and on those treated with oxytetracycline, the predominant spoilage organisms were nonpigmented and pigmented pseudomonads, the former outnumbering the latter. However, with chlortetracycline, the *Pseudomonas* strains were at first completely restrained, the main spoilage organisms at a 'slight off odour' stage being yeasts and strains of *Achromobacter*, though pigmented pseudomonads had developed at the later stage of 'strong off odour'. No detailed analysis was made of the spoilage flora of the tetracycline-treated carcases, but they were examined under ultraviolet light for fluorescence. At 'slight off odour' these carcases were markedly more fluorescent than the oxytetracycline-treated carcases, whereas the chlortetracycline-treated carcases showed no fluorescence; this suggests that pigmented pseudomonads were the predominant organisms on the tetracycline-treated carcases. The differences between the antibiotics may possibly be explained by a greater activity of the chlortetracycline, which was able at first to inhibit the growth of all the pseudomonads; when pseudomonads did develop (at 'strong off odour') they were pigmented strains, which have on the whole a greater natural resistance to the tetracyclines. Of 71 strains of pigmented pseudomonads isolated from untreated carcases and the general factory environment 51 grew on agar plates in the presence of 10 μg tablets of oxytetracycline, whereas only 15 strains of non-pigmented pseudomonads were resistant out of 69 tested.

From these results one might anticipate that the tetracycline antibiotics would have less effect in a processing plant where the predominant spoilage organisms were the pigmented rather than the nonpigmented pseudomonads. This might explain some of the differences observed when the same antibiotics have been tested in different plants. In a general survey of the processing plant concerned here, it was shown (Barnes, 1960) that psychrophilic *Pseudomonas* strains were present on the equipment, in the water, and especially in the ice slush; and they reached the carcases mainly by contact infection from these sources. No such organisms could be found in gut contents from the chickens. When so many of the pseudomonads are resistant to low levels of the tetracyclines, prolonged use of the antibiotic will probably lead to a selection of the more resistant types, particularly in the ice slush tanks where they multiply. Only stringent hygienic precautions can prevent this.

Achromobacter strains, on the other hand, were found to enter the plant in large numbers on the feet and feathers of the birds, there being about 10^8/g of feathers (Barnes, 1960). These organisms too were not found in the gut, but seemed to get on to the feathers from the deep litter on which the birds had been reared. All these birds had been fed on a diet containing 5 p/m of chlortetracycline, so there would be trace amounts of the antibiotic in the deep litter, from the voided faeces and from the food itself. However, of 33 strains of *Achromobacter* isolated from 4 samples of deep litter none was resistant to 10 μg of oxytetracycline; only 4 strains out of a total of 98 isolated from different parts of the poultry plant and from the processed birds themselves were resistant; and none of the 12 strains isolated from the chlortetracycline-treated carcases before storage was resistant. Nevertheless, when the chlortetracycline-treated carcases had a slight off odour, strains of *Achromobacter* predominated together with yeasts, while no pseudomonads were isolated; and, of 40 strains of *Achromobacter* isolated from the oxytetracycline- or chlortetracycline-treated carcases after storage, 35 were resistant to 10 μg of oxytetracycline. No other differences were found to distinguish these strains from the sensitive ones on the control carcases. The origin of the resistant strains is not known, though so far no *in vitro* tests have been carried out to determine how quickly *Achromobacter* spp. can become resistant to these antibiotics.

As a general conclusion it may be stated that the use of the tetracycline antibiotics, in particular chlortetracycline, alters the *Pseudomonas–Achromobacter* spoilage flora of refrigerated stored poultry. Not only do resistant strains of *Achromobacter* develop, but the normally more sensitive nonpigmented pseudomonads may be replaced by the less sensitive pigmented strains.

4. EFFECT OF IRRADIATION

The initial effect of irradiation, unlike that of the bacteriostatic antibiotics discussed, is to kill a large proportion of the micro-organisms initially present, and the increased storage life obtained depends on the fact that the survivors naturally take longer to reach the numbers causing spoilage. Several factors may be involved here, in addition to the actual reduction in numbers of viable organisms. First, there is the possibility that chemical changes caused in the food by radiation may retard the growth of micro-organisms: this has not so far been examined. Second, there is the fact that the 'survivors' which eventually multiply may be damaged by the radiation and hence have a longer lag period than normal, and third, the normal spoilage flora may be more sensitive to radiation than other organisms present and therefore the type of spoilage may be changed.

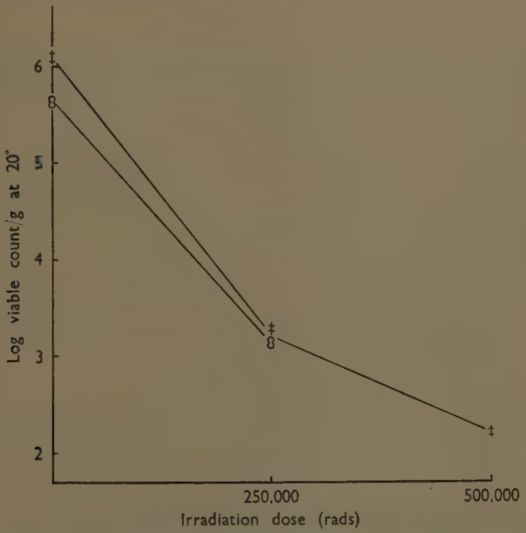

Fig. 2. The effects of chlortetracycline and irradiation with Co 60 gamma rays on the survival of bacteria in whole eviscerated chicken. Crosses, no chlortetracycline; circles, chlortetracycline dip containing 10 p/m.

This last consideration is the one which chiefly concerns us here. The initial effects of the radiation are seen in Fig. 2, which illustrates an experiment where both radiation and chlortetracycline were used on chicken carcases from the modern plant mentioned above. These experiments were described by Ingram & Thornley (1959). Whether or not an antibiotic was present made very little difference at this initial stage, the 250,000 rads dose decreasing the numbers of viable organisms by a factor of about 10^3. There was relatively less mortality, however, on doubling the dose to 500,000 rads, and the change was found to be due to the persistence of a small fraction of more resistant organisms. Fig. 3 shows that the initial flora was of pigmented *Pseudomonas*, *Achromobacter* and a miscellany of other organisms; after 250,000 rads these had given place to about 10% *Achromobacter* and 90% yeasts, while after 500,000 rads, yeasts were the only organisms isolated. It is generally agreed that *Pseudomonas* are especially sensitive to radiation, while yeasts are more resistant than most bacteria (Dunn *et al.* 1948; Wolin, Evans & Niven, 1957).

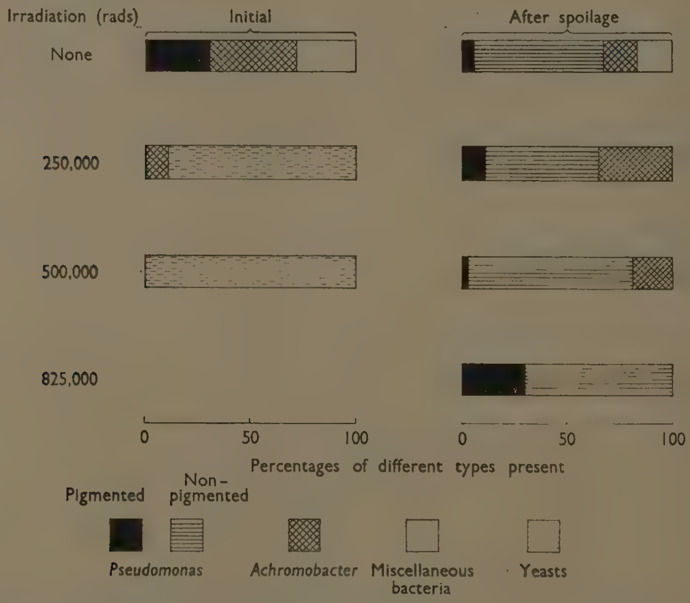

Fig. 3. The effects of irradiation and storage at 1° on the micro-organisms present on chicken carcases.

The flora after spoilage at 1° is shown in the right hand side of Fig. 3. As already described, nonpigmented *Pseudomonas* were predominant on the controls, but it was surprising to find that they were also the most numerous group when spoilage eventually took place on the irradiated birds. Pigmented *Pseudomonas* were also present and formed 29% of the flora on the carcases irradiated with 825,000 rads, whereas *Achromobacter* were relatively more numerous (37%) after 250,000 rads. In all these cases, however, *Pseudomonas* spp. were sufficiently dominant to impart the usual putrid smells to the spoiled carcases.

Two further experiments gave results contrasting with these, but in both cases the poultry were from the farm-scale plant using dry evisceration and storage was at 3° instead of 1°. Fig. 4 shows the results of the first experiments, in which the predominant bacteria were not identified but a preliminary classification was made using the antibiotic screening tests of Shewan, Hodgkiss & Liston (1954). The control carcases, when spoiled, carried mainly penicillin-resistant, oxytetracycline-sensitive strains, of which the majority proved to be nonpigmented *Pseudomonas*, as usual. All the irradiated carcases, when spoiled, had largely peniclin sensitive, oxytetracycline sensitive strains which proved to be *Achromobacter*; and in this case the time of spoilage was very difficult to determine, as there were sweetish, fruity odours in place of the usual putrid smells.

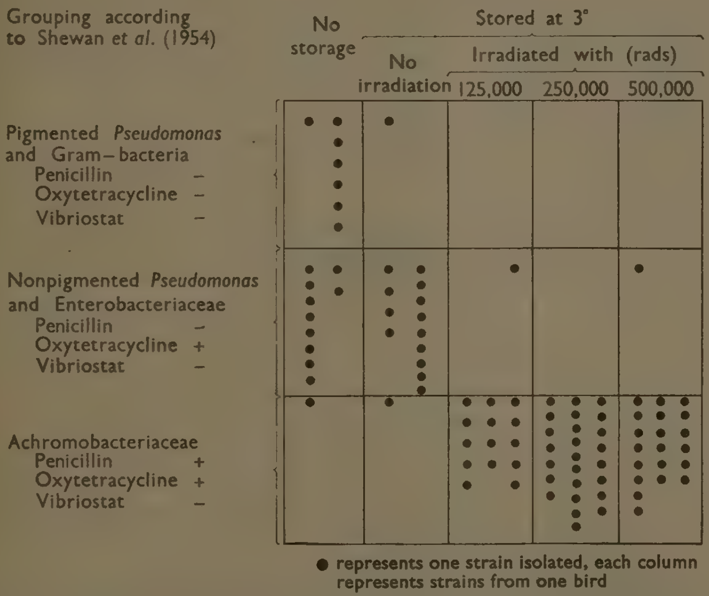

Fig. 4. The effects of irradiation with Co 60 gamma rays and storage at 3° on the types of bacteria present on eviscerated chicken carcases. Resistant, –; sensitive, +.

The second experiment. done in the same poultry plant. differed somewhat in that the controls after spoilage showed mainly *Achromobacter* (50%) whereas the irradiated carcases contained 77% *Achromobacter*. On two irradiated carcases, however, Gram-positive organisms similar to *Lactobacillus* (Thornley & Sharpe, 1960) became predominant and these produced cheesy smells.

As the results following irradiation depend on the survival of small fractions of the initial flora, it is reasonable to presume that these differences may be related to the source of supply of the carcases. In the experiments where pseudomonads eventually predominated on the spoiled irradiated carcases, the poultry plant was of the modern type, using hot evisceration with copious washing: Barnes (1960) has shown that in this factory the pseudomonads originated in the water supply. In the other two experiments, where cold evisceration was done by hand, no washing was used: therefore the proportion of *Pseudomonas* to *Achromobacter* carried on the carcase was likely to be much lower, and this may well have caused the different results.

5. THE COMBINED EFFECTS OF CHLORTETRACYCLINE AND IRRADIATION

This combination of treatments was included in the experiment at the modern poultry plant. Carcases were dipped in 10 p/m of chlortetracycline and then irradiated with 250,000 rads. The initial flora was the same as that found after the radiation treatment alone, i.e. 10% *Achromobacter* and 90% yeasts (Fig. 5). It will be recalled that chlortetracycline treatment alone resulted in spoilage by a mixture of yeasts and tetracycline-resistant *Achromobacter*. In this case the tetracycline-resistant *Achromobacter* formed 21% of the flora whereas nonpigmented *Pseudomonas* accounted for 76% and pigmented *Pseudomonas* 3%. Nevertheless, there was an extension of storage life roughly equal to that of the sum for the two separate treatments.

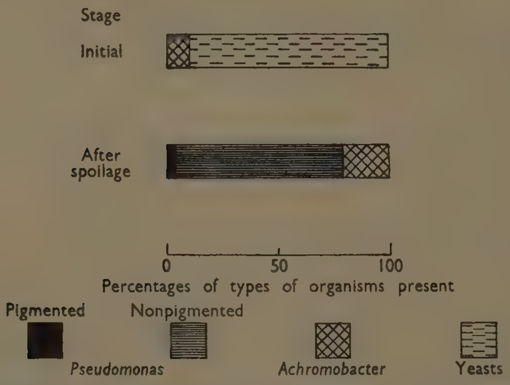

Fig. 5. Effects of chlortetracycline (dip containing 10 p/m) combined with irradiation (250,000 rads) and storage at 1° on the micro-organisms present on chicken carcase.

6. DISCUSSION

With antibiotic treatment or with irradiation, the spoilage flora of the chicken carcase is altered. Both treatments favour the survival of yeasts but these multiply slowly, so that they predominate only in the intermediate period of storage, being eventually outgrown by the bacteria. The yeasts do not produce very obvious changes. If observations are only made when putrid off odours appear, putrefying bacteria will by then have become dominant. Different opinions about the relative importance of yeasts and bacteria may obviously have arisen through observations made at different stages in spoilage.

As regards the bacteria themselves, a simple view based mainly on laboratory experience would be that *Pseudomonas* is most sensitive to radiation but that *Achromobacter* is more sensitive to tetracyclines than *Pseudomonas*, especially the pigmented strains (*cf.* the antibiotic screening test of Shewan *et al.* 1954).

In our antibiotic experiments the ultimate spoilage flora was in fact generally dominated by pseudomonads, but there were significant differences between the three tetracycline compounds. Chlortetracycline almost eliminated the pseudomonads, only pigmented strains appearing at a later stage; with tetracycline, to judge from the ultraviolet fluorescence, pigmented strains were abundant; with oxytetracycline, nonpigmented strains dominated throughout, though growth was delayed. The picture was complicated, however, by the appearance, with both the tetracyclines investigated in detail, of a resistant group of *Achromobacter* spp. whose origin is still uncertain. They entirely displaced pseudomonads on the chlortetracycline-treated chickens at 'slight off odour'. With oxytetracycline, too, these organisms developed but even at 'slight off odour' they did not predominate.

Whereas these antibiotics only exert a bacteriostatic effect, radiation destroys organisms and might be expected to have a greater effect on *Pseudomonas* than on *Achromobacter*. Thus a combined treatment should be highly effective. In our experiment, the extension in storage was indeed approximately the sum of those from the two treatments separately, but it was surprising to find that on eventual spoilage nonpigmented pseudomonads predominated. This finding agrees with that of Shewan (1959) for fish treated with chlortetracycline and 250,000 rads, where pseudomonads eventually developed at 0°, although the flora immediately after treatment contained 92% of yeasts.

The expected result was found in experiments with dry-eviscerated poultry, where moderate doses of irradiation killed the pseudomonads and spoilage occurred with *Achromobacter* which, as they do not produce amino compounds so readily, did not give rise to the usual putrid smells. But the pattern was more complicated in the experiments with eviscerated poultry chilled in ice slush, where pseudomonads appeared as an important element in the spoilage flora after irradiation. It is not known whether this was because the carcases were from a factory chilling with ice slush, thus introducing more *Pseudomonas*, or because they were wrapped differently, or stored at a slightly lower temperature, or whether these particular pseudomonads possessed an unusually high resistance to radiation.

Results very similar to those obtained with poultry chilled in ice slush were found by Shewan (1959) with fish, which is also chilled in ice after catching. Although the proportion of *Pseudomonas* to *Achromobacter* was diminished initially by irradiation with 250,000 and 500,000 rads, on eventual spoilage at 0°, pseudomonads were dominant.

From the point of view of this Symposium there is a special interest in the different types of spoilage associated with the different groups of organisms: putrefactive with the *Pseudomonas*; fruity with the *Achromobacter*; certainly slower and perhaps lipolytic with the yeasts. These differences are apparently the reflection of systematic differences in metabolism between the several groups of organisms which, one presumes, might help in classifying the organisms, and furthermore in a manner likely to be helpful in practice. The arginine test is an apt illustration of this point: it neatly separates *Pseudomonas* from *Achromobacter*, and it was developed from the observation that only the former produces ammonia from peptone under conditions of restricted air supply (Thornley, 1960), an observation which clearly parallels the spoilage patterns associated with these bacteria.

Such differences are of practical importance too because, if antibiotic or irradiation processing were adopted, the consumer would evidently have to learn to recognize new criteria of spoilage which, at present, we are not in a position to define adequately

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper VIII)

MECHANISM OF THE MULTIPLICATION OF *PSEUDOMONAS* IN THE HEN'S EGG

By (the late) J. BROOKS

Low Temperature Research Station, Downing Street, Cambridge

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1. Introduction

It has long been known that egg white *in vitro* is not a favourable medium for the growth of bacteria. Reasons that have been suggested to explain this fact were listed twenty years ago by Haines (1939). They were: first, the presence of anti-bacterial substances such as lysozyme in the white; second, the high pH of the white—about 9·3; and third, the unavailability of the native proteins for bacterial nutrition. Since that time the following additional reasons have been suggested: the presence in the white of conalbumin which, by forming a firm complex with iron, renders most of this metal unavailable to the bacteria; and the presence of the specific protein avidin, which does the same thing with biotin.

The structure of the hen's egg is shown in Fig. 1. On the average, nearly one-half of the white of a newly laid egg consists of a gel, the thick white (K), interposed between two liquid fractions, the outer thin white (J) and the inner thin white (L). The two shell membranes (H) separate at the broad end of the egg to enclose the air cell (A).

The contents of about 90% or even more of newly laid eggs are free from micro-organisms and it has been customary to speak of the egg as possessing natural defences against bacterial attack. Naturally, one suggested defence was the mechanical protection provided by the shell against bacterial invasion. Another was afforded by the properties of the egg white described above. Finally, it was thought that the two shell membranes which separate the white from the shell not only afforded some mechanical protection but actually possessed bactericidal properties. Although these suggestions were very reasonable there was little or no direct experimental evidence for some of them.

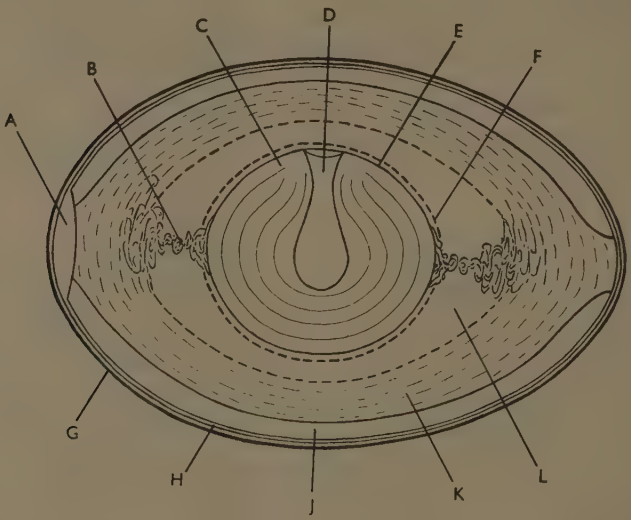

Fig. 1. The structure of the hen's egg, as shown by a section through the long axis (Brooks & Hale, 1959).
A, air cell; B, chalazae; C, yolk; D, germinal disc and white yolk; E, vitelline membrane; F, film of mucin; G, shell; H, shell membranes; J, outer thin white; K, thick white; L, inner thin white.

In work to be described (Brooks & Hale, 1961*a*,*b*,*c*) suspensions of a pure culture of a pigmented *Pseudomonas* were injected into the air cell, and the subsequent accumulation of organisms in the white was measured. By this means any defence offered by the shell was bypassed, but it was also ensured that the injected bacteria still had one shell membrane to cross before reaching the white. Bacteria which penetrate the shell naturally (and we think that this only occurs when the outside of the shell is wetted or washed) have to penetrate two shell membranes. Both shell membranes, however, are composed of mucin and keratin fibres.

2. The Effect of Age on the Multiplication of *Pseudomonas* in the Egg

This work was started because of an observation made during earlier work. We noticed that when injected eggs were kept at 20°, the age of the egg at injection seemed to have a large effect on the subsequent rate of bacterial accumulation in the white. The older the egg at injection, the more rapidly did bacteria accumulate within it.

(a) *Technical difficulties*

There were two technical problems in this work. One was to devise an aseptic technique for handling the eggs and the material obtained from them. The use of naturally clean eggs from a well managed farm, and a large Perspex dissection chamber with two portholes fitted with shoulder length rubber gloves, solved this problem. The second was more troublesome: namely, how to preserve the various cultures in such a manner that their growth rate in a given medium, say in fresh egg white *in vitro*, did not change from one week to the next. Freeze drying and storage of the dried material at 0° proved to be unsatisfactory. This treatment may not have changed the biochemical characteristics of the organisms but it certainly altered their kinetic behaviour.

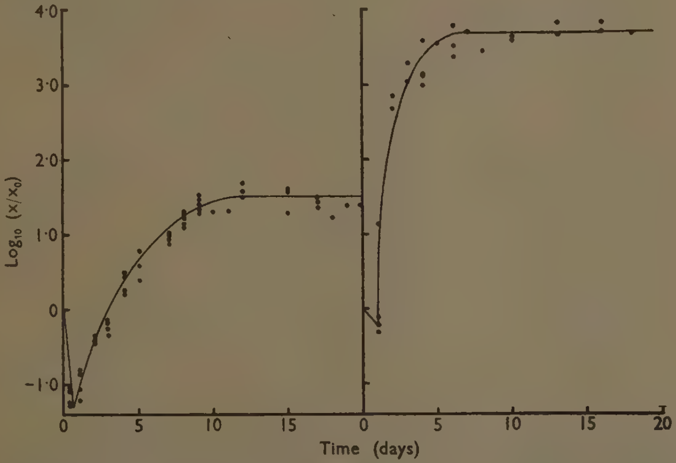

Fig. 2. The growth *in vitro* of two cultures of *Pseudomonas* in the white of newly laid eggs in different experiments over a period of several months.
x_0, original number of bacteria; x, number of bacteria when sampled.

The method finally used resembled that described by Rhodes (1956) in which a stock culture of the organism on a slope of casein digest agar was kept almost completely covered by medicinal paraffin. Organisms from this slope, after subculturing on several successive days, gave suspensions containing 90% or more of viable organisms and with sufficiently reproducible growth rates. The behaviour of two strains is shown in Fig. 2. In both instances, the values refer to growth *in vitro* in the white of newly laid eggs using an initial inoculum of from 10^5–2×10^5 viable cells/ml of white and to experiments made at different times over a period of several months.

(b) *Pattern of multiplication*

To determine the effect of the age of the egg on the rate of growth of *Pseudomonas*, several pigmented cultures were used. They had all originally been isolated from eggs and, incidentally, none was sensitive to lysozyme. Two of the cultures were named species (*Ps. schuylkilliensis* and *Ps. putida*) and the others were numbered, but all showed similarly the effect of the age of the egg when injected.

The type of result obtained with one culture is shown in Fig. 3. Curve A is from an *in vitro* experiment. Here, newly laid eggs were broken open, the whites mixed and about $1{\cdot}5 \times 10^5$ viable cells/ml of white added. The initial load was comparable with that in the injected eggs. There, about 5×10^6 viable cells were injected into the air cell of each egg. As each egg contains about 35 ml of white, this corresponds, as in the flask, to about $1{\cdot}5 \times 10^5$ cells/ml of white. It will be seen that *in vitro* the organisms, after an initial kill, multiplied feebly and reached an approximately steady level at less than 10^7/ml, whereas in the white of the intact egg, counts from 10^9 to 10^{11}/ml were obtained, depending on the age of the egg when injected. However, in the egg inoculated at 24 hr there was a sudden acceleration in rate at about the 13th day. Other changes, which will be described later, also occur at about this time.

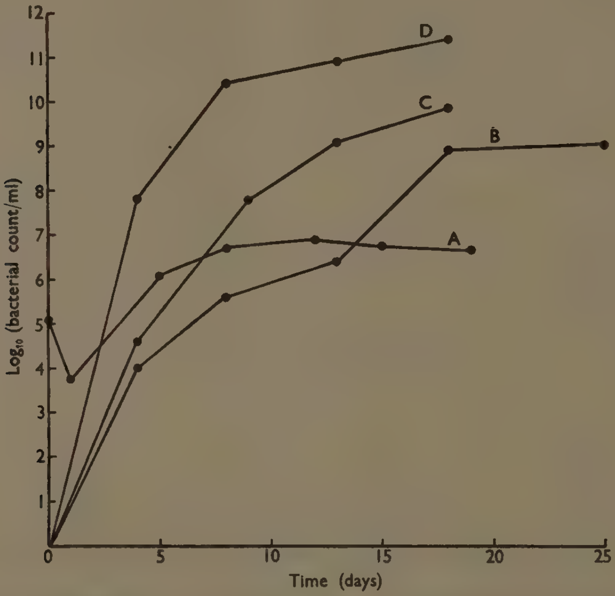

Fig. 3. The effect of the age of the egg on the multiplication of *Pseudomonas* in the white.
A, white *in vitro*; inoculated within 24 hr of laying.
B, C and D, white in eggs; B, injected within 24 hr of laying, C, injected one week and D, injected 2 weeks after laying.

The growth of *Pseudomonas* in both the white and the yolk of eggs injected 24 hr, 2 weeks and 4 weeks after laying is shown in Fig. 4. There was little difference between the 2 and 4 weeks' old eggs, and this type of result was general. Increasing

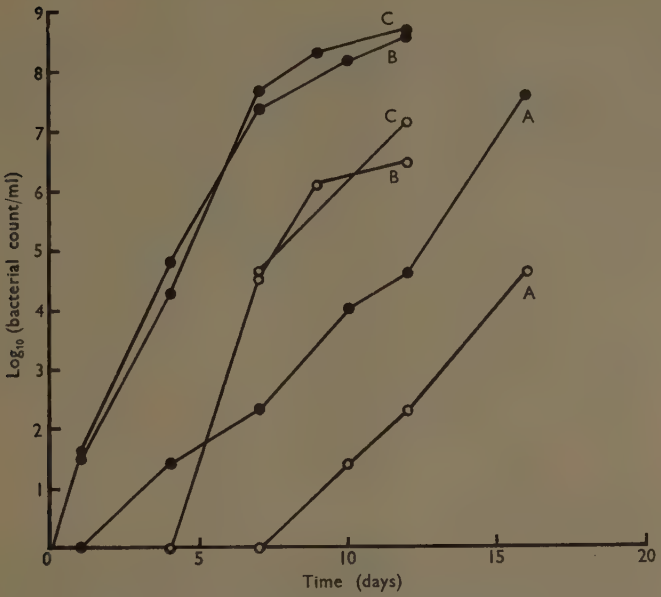

Fig. 4. The effect of the age of the egg on the multiplication of *Pseudomonas* in the white (full circles) and the yolk (open circles) of eggs. A, eggs injected within 24 hr of laying; B and C, eggs injected 2 weeks and 4 weeks after laying.

the gap between laying and injecting from 24 hr to 1 week had the greatest effect. Injecting 2 weeks after laying increased the rate of accumulation of bacteria in the white still further but to a less extent. Increasing the gap to 3 or 4 weeks had little further effect. Fig. 4 also shows that the rate of accumulation suddenly increased when eggs injected soon after laying were kept for about 12 days. The yolk curves have the same shape as the corresponding white curves but are displaced towards the right, which can be explained by supposing that no bacteria enter the yolk until a fair number have accumulated in the white.

3. POSSIBLE EXPLANATIONS FOR THE DECREASING RESISTANCE OF AGING EGGS TO BACTERIAL ATTACK

(a) *The effect of age on the ability of the white to sustain bacterial growth*

How can we account for the decrease in resistance to bacterial attack as the egg ages? At first sight, the factors which have already been mentioned as limiting bacterial growth in the white do not seem as if they would be affected by age. For example, the pH of the white of the intact eggs remains remarkably constant for long periods. It seemed possible, however, that essential nutrients might be scarce in the white of newly laid eggs but might enter the white from the yolk when the eggs were kept. The yolk contains plenty of iron, inorganic phosphate, free amino acids and biotin, whereas the newly laid white contains little or none of these. Further, it is known that some of these substances diffuse from the yolk to the white when eggs are kept, although the rate of diffusion is low. The scheme shown in Fig. 5 was therefore devised.

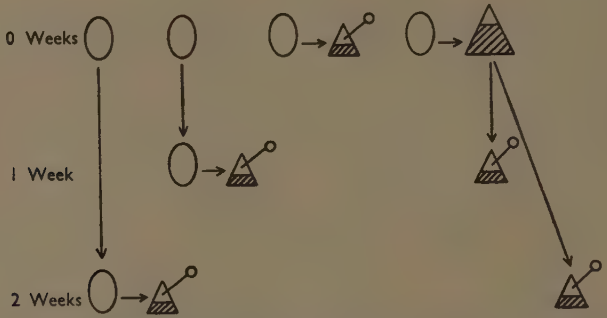

Fig. 5. The scheme of experiment to determine the influence of the diffusion of essential nutrients from the yolk to the white on the multiplication of *Pseudomonas* in the white (see text).

A large batch of newly laid eggs was randomized. Some of them were broken open and the mixed white divided between a number of flasks. The rate of multiplication of one of the *Pseudomonas* cultures in the white was then measured. The other flasks were kept at 20°, inoculated at intervals and the rate of multiplication measured. Over the same period intact eggs were being kept. These were broken open at intervals, the white inoculated *in vitro* and the rates of multiplication measured, so that the properties of white kept *in vitro* were being compared with those of white kept in intact eggs. In the former, there could be no diffusion of nutrients into the white from the yolk; in the latter, there could be. It was found that age had little or no effect on the ability of the white to sustain bacterial growth, regardless of whether the white had been kept *in vitro* or in the intact egg. Hence it seems improbable that the operative factor could be the diffusion of nutrients from the yolk into the white.

(b) *The effect of age on the ability of the air cell membrane to support bacterial growth*

To return now to the effect of the age of the egg when injected. Examination of broken-out, injected eggs in ultraviolet light after keeping them at 20° showed that the air cell membrane, far from possessing bactericidal properties seemed, judging by the intense fluorescence of an ever increasing area on the membrane, to be supporting a vigorous growth. Reports also appeared in the U.S.A. (e.g. Stokes &

Osborne, 1956; Garibaldi & Stokes, 1958) which stated that the shell membranes could sometimes encourage growth rather than hinder it.

Fig. 6 shows the results of *in vitro* experiments in which the rate of multiplication of one of the *Pseudomonas* cultures was measured in egg white from newly laid eggs and from eggs 26 days old in the absence and presence of one-half of a shell membrane stripped from an egg. There can be no doubt that the presence of the membrane accelerated the rate of multiplication of the organisms. It was further shown that membrane from an egg which had been kept accelerated the rate of growth to a greater extent than membrane from a newly laid egg.

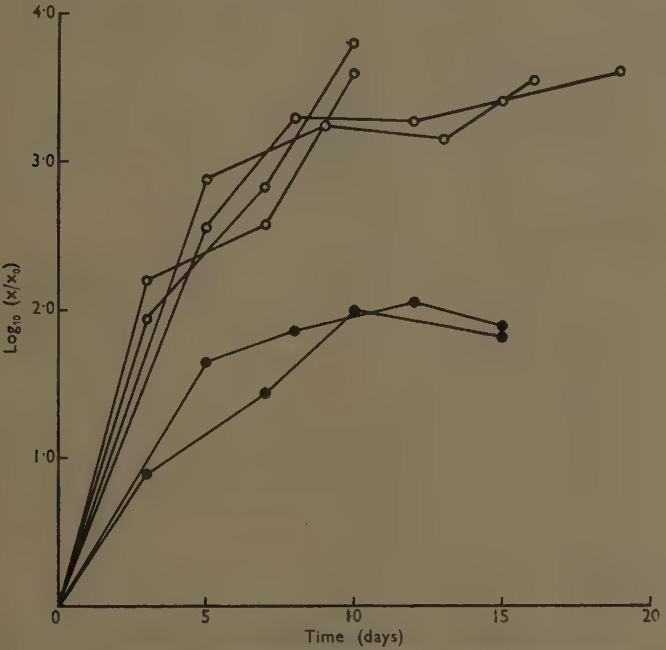

Fig. 6. The influence of added air cell membrane on the multiplication of *Pseudomonas* in egg white *in vitro*.
Full circles, multiplication in new or old white in the absence of added membrane; open circles, multiplication in various combinations of new or old white with membrane from new or old eggs. x_0, original number of bacteria; x, number of bacteria when sampled.

The next series of experiments resembled those which have already been described. About 5×10^6 viable cells were injected into the air cell of eggs of different ages and the rate of accumulation of cells in the white was measured. In these experiments, however, the air cell membranes were also excised when the eggs were broken open. The membranes were freed from adhering white and loosely attached cells by rinsing in quarter-strength Ringer's solution, disintegrated with a high speed blender in more of that solution, and a count made on the suspension.

The results with one particular species are shown in Fig. 7. There are several points of interest in these curves. Considering the membrane curves, the bacteria multiplied more rapidly from the start on the membrane of the 22 days old eggs.

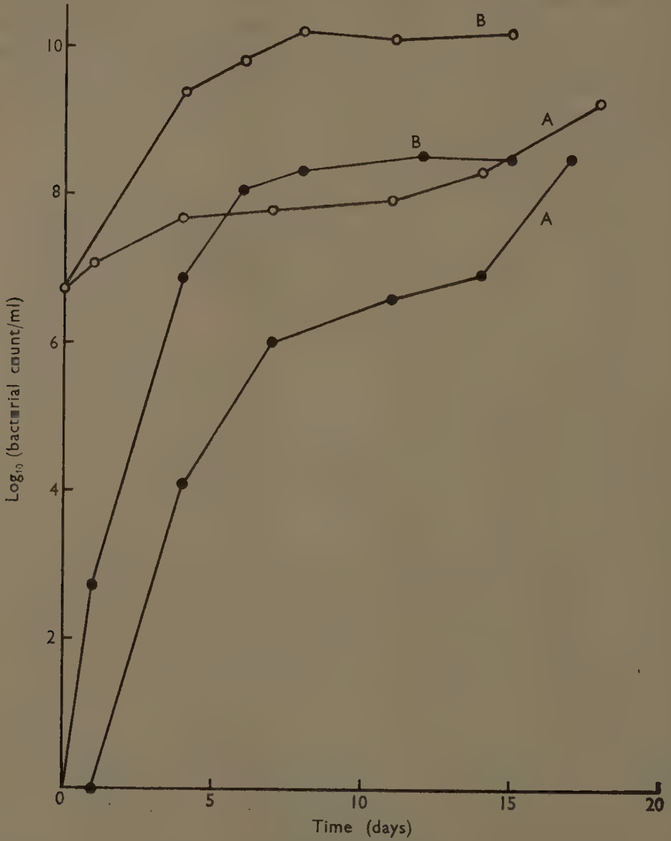

Fig. 7. The effect of the age of the egg on the multiplication of *Pseudomonas* in the white (full circles) and the air cell membrane (open circles). A, multiplication in eggs injected within 24 hr of laying; B, multiplication in eggs injected 22 days after laying.

With the eggs injected within 24 hr of laying, the rate of multiplication on the membrane was low initially but began to increase rather suddenly when the injected eggs were kept for more than 12 days. Further, this increase of rate was paralleled by an increase in the rate of accumulation of bacteria in the white at about the same time. This correspondence strongly suggests that many, if not most, of the bacteria in the white were simply invaders from the membrane, and that the rate of their subsequent multiplication in the white was unimportant in comparison with the rate of invasion.

As previously described, 10 different cultures of pigmented *Pseudomonas* were used and five of them were studied in the detail described above. They behaved differently, but the differences could plausibly be attributed to the different ways in which the cells of a given culture grew on and attacked the membrane. Some cultures grew rapidly and cells began to appear in the white soon after injection, whereas others grew more slowly and cells did not appear in the white until several days had elapsed. For example, cells of one culture, when injected into the air cell of newly laid eggs, did not appear in the white until 6 days had elapsed; cells of the same culture injected into the air cell of 16 days old eggs first appeared in the white 3 days after injection.

With these data an attempt was made to calculate the number of bacteria which had migrated to the white directly from the membrane and to compare this with the number which subsequently developed in the white. The results of the calculations suggest that nearly all the bacteria which accumulated in the white were simply invaders; that the number of bacteria which penetrated the membrane in unit time rapidly increased as time elapsed; and that, from the start, the membrane of an old egg allowed more bacteria to pass into the white than the membrane of a newly laid egg. Further, the membrane before it is exposed to bacterial attack is impermeable to bacteria, although this is not shown by these experiments.

These results explain the effect of the age of the egg when injected on the subsequent accumulation of cells in the white. The difference between newly laid and older eggs is governed by the effect of age on the properties of the shell membranes. Our results show that with time the membrane undergoes a change which has two effects: first, the organisms multiply faster and farther upon it, and second, the organisms penetrate it more easily. Expressed in a different way, it might be said that, with time, a spontaneous change occurs in the membrane which makes it more susceptible to bacterial attack. Moreover, there is an indication that this change in the properties of the membrane becomes more rapid at about the 12th day.

4. THE NATURE OF THE NUTRIENT WHICH LIMITS MULTIPLICATION OF *PSEUDOMONAS*

Unfortunately we do not know what causes the above mentioned change with time in the properties of the shell membrane, but we have tried to find out why the membrane is a more favourable medium for bacterial growth than the white, and why the white itself is such an unfavourable medium. American workers (e.g. Stokes & Osborne, 1956) have suggested that membrane added to a suspension of cells in the white accelerates bacterial growth because it provides organic nutrients which are absent from the white or present in limited amounts only. We do not think that this is the cause, and base this conclusion on the fact that we found that the addition of glycine or ammonium salts to the white *in vitro* had no effect on the rate of multiplication of *Pseudomonas* cultures. The white, incidentally, does not lack an adequate source of carbon for bacterial nutrition as it contains about 0·4% of glucose. We think, in fact, that the important primary factor is the available supply of iron and, possibly, of other trace elements.

To consider first the white, Waring & Werkman (1942) and Fraenkel-Conrat & Feeney (1960) state that *Pseudomonas* cultures require iron at 0·1 μg/ml for optimum growth. The total concentration of iron in the white is often given as 1–2 μg/ml, but some or most of this is believed to be firmly bound by the conalbumin. However, using more modern methods of analysis, we have found that the concentration of total iron in the white is much less than was formerly supposed, the average being only 0·15 μg/ml and the range 0·1–0·25 μg/ml. When the presence of conalbumin is taken into account, there seems little doubt that the white is short of available iron. Further, by using the method of paper chromatography, we have found that apart from copper (which also forms a complex with conalbumin), other heavy metals are either absent from the white or present in concentrations apparently even smaller than that of iron. The shell membrane, on the other hand, was found to contain a rich variety of trace elements: aluminium, cobalt, copper, iron, nickel, tin and zinc were easily identified. Moreover, the iron content of the shell membrane was high compared with that of the white. If concentrations are expressed in terms of Fe/g dry weight, that for the membranes was 15 μg/g, whereas that for the white was 1·3 μg/g.

5. THE INFLUENCE OF PH ON THE IRON-CONALBUMIN COMPLEX

If growth in the white is limited by a shortage of available iron, can it be increased by adding more iron to the white? It is known that if more iron is added much of it will be firmly bound by the conalbumin. We found no acceleration when the total iron concentration in the white *in vitro* was raised to 2, 3 or 4 μg/ml, but there was a considerable increase in growth rate at 20 μg/ml. Under ordinary circumstances, for instance in a synthetic medium, such a high concentration is said to depress the growth rate.

Considering the iron-conalbumin complex, one would expect its dissociation constant to be dependent on pH. Thus, if the pH of the white were lowered by the addition of acid, less of the total iron present should be bound to the conalbumin. It seemed possible, therefore, that an acceleration in growth rate caused by the addition of iron might be more evident at a pH lower than that of the white in its natural state (about 9·3). We found, in fact, that when the pH of the white was lowered to 6·0 the addition of 2 or 3 μg of iron/ml of white increased the rate of growth of *Pseudomonas* considerably, compared with the rate in the white alone at pH 6·0.

It was also observed that merely lowering the pH of the white to 6·0 greatly increased the growth rate even without any addition of iron. This increase might be thought of as the sum of two effects; the effect of pH alone on bacterial growth plus the effect of the extra iron made available by the increased dissociation of the iron-conalbumin complex also caused by the lowered pH. Clearly, it was necessary to compare the effect of altering the pH of a synthetic medium which contained no conalbumin or other compounds capable of firmly binding iron.

The synthetic medium used contained glycine and glucose as nitrogen and carbon sources, phosphate, carbonate and bicarbonate to buffer it over the whole pH range from 9·5 to 6·0, Na, K and Mg, and traces of Zn, Cu, Mn, and iodide ion. Even when analytical grade chemicals were used there was enough adventitious iron present in this medium to give a very rapid rate of growth of *Pseudomonas*, even at pH 9·0. However, by a preliminary treatment of the reagents with a powerful chelating agent, 8-hydroxyquinoline in chloroform solution, we were able to obtain a synthetic medium free from the chelating agent which, at pH 9·0, gave a rate of growth similar to that in egg white at the same pH.

The next step was to alter the pH of both the white and the synthetic medium by the addition of various amounts of acid, and then to determine the rates of multiplication of *Pseudomonas* in the two series of media whose pH ranged from 9 to 6·5. In both series, the rate of multiplication was the greater, the lower the pH; but lowering the pH of the white increased the rate to a very much greater extent than a similar lowering of the pH of the synthetic medium. This result furnishes indirect support for the suggestion that one of the primary factors limiting the rate of growth in the white is a shortage of available iron. We do not think it is the only factor, because we never succeeded by the addition of iron alone in raising the rate of growth to a value equal to that which could be obtained in a favourable medium. Even when the lack of iron is wholly or partly remedied, the rate of growth in the white, although increased, might still be limited by some other factor; for instance, an inadequate source of nitrogen.

Finally, it was mentioned earlier that when a suspension of bacteria was added to the white *in vitro*, some of them were killed. This effect is apparently not caused by the presence of lysozyme in the white. In general, the extent of the kill seemed to be related to the subsequent rate of growth, the kill being less with the more rapidly growing species. It was observed that a similar or even greater kill occurred at pH 9·0 when cells were added to a synthetic medium which contained less than 0·1 μg/ of iron/ml. On the other hand, the extent of the kill was much less in the same synthetic medium if it contained enough iron to give a rapid rate of growth.

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper IX)

THE DISTRIBUTION AND ECOLOGY OF GRAM-NEGATIVE ORGANISMS OTHER THAN ENTEROBACTERIACEAE IN LAKES

BY VERA G. COLLINS

Freshwater Biological Association, Ambleside, Westmorland

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1. INTRODUCTION

THE MAIN objective of this investigation has been to show that both mud and water bacteriology can never be solely a study of bacterial physiology, but that the problems are largely ecological in nature.

The work, carried out over the past few years, has involved the examination of routine weekly samples from two lakes in the English Lake District, namely Windermere and Esthwaite Water, and also of monthly samples from the surface waters of every other lake in the District. The results present a composite picture of the numbers and types of bacteria in these lake waters throughout the seasons, and from them it has been possible to relate the bacteriological conditions to the water movements, with particular emphasis on the effects of temperature and rainfall, and also to consider the influence of the main physical factors of the periods of stratification and overturn.

2. BACTERIA IN LAKE WATERS

The bacteria present at any time in the water of a lake may be considered in three categories: those washed in from outside sources, of which a large proportion will ultimately perish but some will find conditions suitable for growth; indigenous types capable of existence in a dilute nutrient solution as represented by lake water, and able to use for growth low concentrations of available organic matter; and those dependent on a solid surface for their proliferation and therefore found in connection with soil particles, mud detritus and plankton. This last group may be divided further into types with special hold-fast mechanisms, such as stalks, and found only under natural conditions, and others which adhere to surfaces by mucilaginous material and grow on the sides of containers.

(a) *Seasonal changes*

Although the present investigation is by no means complete, and further experiments are required to clarify effects due to seasonal changes in the phytoplankton and chemical composition of the lake water, it is believed that a general picture of the bacteriology of lake water can be drawn as follows: normally growth is limited by the supply of available carbon and energy sources; therefore, during long rainless periods the bacterial counts decrease steadily and only increase in surface waters at the onset of maximum temperature conditions and also after heavy rain. This increase is due partly to washed-in bacteria and partly to multiplication of types which avail themselves of the washed-in organic matter. The water acts as a selective agent in determining the types of washed-in soil bacteria which survive in this medium, some of the most common soil bacteria being absent from lake water. Factors which affect the selection of different types of bacteria include the concentration of dissolved substances, particularly phosphates, the availability of organic nitrogen for types which are unable to use inorganic nitrogen, and the presence or absence of particulate matter necessary for certain types of bacteria which require a site for attachment.

(b) *Effect of stratification and the overturn*

In the two lakes mentioned, the numbers of bacteria decreased at all depths from the onset of stratification in May until October and the overturn. The only feature of special note during this period concerned the effect of a short period of heavy rainfall on the numbers and types of bacteria at two critical layers in the lakes; it was reproduced in both cases, but more strikingly in Windermere, during a few weeks of very calm weather with negligible wind effects. The effect was that of an apparent concentration of bacteria on the thermocline. In the water above the thermocline, plate counts gave 2,400 bacteria/ml, but at the top of the thermocline 47,700 bacteria/ml were found. The numbers decreased through the thermocline to 1,460 bacteria/ml and then increased again until there were 60,240 bacteria/ml at the bottom of the thermocline. Below that level the numbers again steadily decreased to a depth of 60 m, the lowest depth sampled.

The types of bacteria isolated included some of those normally found in the main inflow into the north basin of the lake, and were detectable as sewage organisms, but the greater proportion were types usually associated with soil particles brought down into the lake by heavy rainfall. It would appear, from this effect, either that the increased volume of the cooler river water, bringing with it all the detritus associated with flood water and heavy rainfall, had slid under the warm surface layers of the lake, thereby enriching and changing the bacterial flora of these layers, or that, quite independent of the effect of rainfall during calm weather, there was a large 'sump' of material in the form of phytoplankton and zooplankton buoyed up at these layers (because of the density difference caused by the temperature gradient), and that this plankton population, rich in organic matter, served as a nutrient food store for the bacteria in the water.

In both lakes the period of overturn during the autumn months, when the water is freely circulating, produced high plate counts at the lower depths; this indicates a release through circulation of the fine particulate matter on the surface of the lake mud, with its accompanying bacterial flora, enriched by the decomposition of algal detritus deposited there during the preceding period of stratification. At no time during this period was there any numerical increase in bacteria, as shown by either direct count or plate count, in the surface layers of the lakes, but what was significant was the change in the types of bacterial colonies which developed. Types were recovered in surface water samples which at other times of the year were present only in the samples from the greatest depths of each lake. This further indicates the effect of circulation of the water on the dispersal of bacteria from the mud surface and the lower layers of water throughout the body of the lake, a complete mixing effect, confirmed by the lake temperature which is the same from top to bottom. It is interesting that the largest numbers of bacteria occur during the winter months when the lake temperatures are the same throughout (and, incidentally, at their lowest), and when the seasonal rainfall for this particular area is at its highest.

3. Bacteria of Lake Muds

A study of the mud of Esthwaite Water, using enrichment culture methods on samples obtained with a Jenkin surface-mud sampler, has revealed the presence of several groups of bacteria. The classification given below is a tentative one, but it serves to indicate the variety obtainable.

In the immediate surface, aerobic bacteria are found in profusion, and their numbers decrease with depth in the cores, which are from 20–30 cm deep. Some of these bacteria therefore tolerate low tensions of oxygen. Among the bacteria present in the surface levels are those which can produce ammonia from peptone, nutrose and urea; proteolytic organisms which can liquefy gelatin and produce hydrogen sulphide from peptone; denitrifying organisms which reduce nitrate to nitrogen, and some which reduce nitrate to nitrite. Bacteria able to fix nitrogen are also present but they are only feebly active. In tests for nitrification 30 days were needed to give a slightly positive reaction. Other organisms found include a large group which can ferment dextrose, a group which can ferment xylose and a very large group indeed which is able to hydrolyze starch. Cellulose decomposing bacteria are also present, as are chitin digesting types, and there is a small group of organisms which can hydrolyze fat. Below the 4 cm level, large numbers of sulphate reducing bacteria are recovered, which is to be expected from the low oxygen tension of this particular environment.

The following types occur seasonally, according to the amount of hydrogen sulphide present in the water immediately overlying the mud surface: members of the family Thiorhodaceae, the coloured sulphur bacteria, mainly *Chromatium* spp.; *Beggiatoa*, the colourless sulphur bacterium; members of the family Athiorhodaceae, the coloured nonsulphur bacteria, mostly *Rhodopseudomonas* spp., and the lime sulphur organism *Achromatium*. Two other main groups of sulphur organisms, namely the sulphate reducing bacterium *Desulphovibrio desulphuricans*, and the thiosulphate-oxidizing bacterium *Thiobacillus thioparus*, have been studied in some detail. The relative abundance of these two groups of micro-organisms is entirely dependent on the amount of oxygen present in the water overlying the mud surface.

4. A SUGGESTED SEQUENCE OF MICROBIOLOGICAL EVENTS

It may be speculated that the following sequence of microbiological events occurs. When the lake waters stratify during the late spring and early summer, giving rise to relatively stagnant physical conditions in the hypolimnion of the lake, the metabolic activities of the aerobic bacterial flora of the mud surface increase and as a result the oxygen tension of the overlying water is reduced to a minimum. Then the sulphate reducing bacteria start to be active, and this results in the production of hydrogen sulphide, and the formation of ferrous sulphides, on the mud surface and in the overlying water. The hydrogen sulphide diffuses into the overlying water, creating a zone of anaerobiosis. At the interface between this zone and the upper zone containing oxygen, members of the coloured sulphur bacteria develop, mostly species of *Chromatium*. In Esthwaite Water mass growths of these organisms have been recovered from depths of from 9–13 m, the maximum depth of the lake being 15 m, and this shows that in this lake the light intensity must be sufficient to support the growth of these photosynthetic autotrophic organisms at those depths. Above this zone in the lake, and therefore above the thermocline system, where there is oxygen and organic matter in the form of decayed planktonic organisms, members of the family Athiorhodaceae are recovered in mass growth. They also are photosynthetic organisms, requiring organic growth factors, but they vary considerably in their oxygen requirements, some species being aerobic, some anaerobic, and others can tolerate low oxygen tensions. Within this system, during stratification, the activities of the filamentous iron organisms must be superimposed. They are not recovered from the de-oxygenated water immediately above the mud surface, but they are present in very large numbers in the thermocline and above it and offer, by means of their filamentous growth, a matrix of colloidal sheaths impregnated with iron compounds—an ideal site for the attachment of other micro-organisms, especially members of the Athiorhodaceae, already mentioned.

At the onset of overturn conditions in the lake the layered system is dispersed, and with it these microbiological zones. At this time masses of these iron bacteria with their attendant microflora are recovered throughout the entire water profile of the lake.

During the stratification period, the main metabolic function of the coloured sulphur bacteria is to oxidize the sulphide produced by the sulphate reducing bacteria back to sulphate. The sulphur cycle is completed during the overturn by the thiosulphate oxidizing bacteria present in the surface mud and in the water overlying the mud, whose metabolic activities are set in motion by the conditions of dispersal of the anaerobic zone, and the re-oxygenation of the hypolimnion. Following closely on their activity in releasing free sulphur, and found predominantly on the mud surface, are species of *Beggiatoa* and the lime sulphur bacterium *Achromatium*, the former storing sulphur in its filaments and the latter storing both sulphur and calcium carbonate.

5. SUMMARY

The work reviewed in this paper concerns the relationship between the bacteriological conditions and the water movements in lakes.

It is concluded that the numbers of bacteria decrease progressively at all depths during the summer months from the onset of stratification in May until the overturn in October or November, and that at the overturn, when complete mixing occurs, there is an increase in the counts from the deeper samples, suggesting that fine particles deposited on the bottom during the period of stagnation in the summer were being swept into suspension as the water movements increased. This is supported by the fact that whereas there was no increase in bacteria in the surface layers during the overturn, the plates showed that the types present included organisms found at other seasons in the samples from the greatest depths.

The bacterial numbers are highest in the winter, when the lake temperature is the same throughout (and at its lowest) and the rainfall is greatest.

During a period of calm weather with heavy rain in the stratification period a new phenomenon was observed, namely the concentration of bacteria at boundary layers in both lakes.

A tentative classification of the groups of bacteria present in the surface mud of Esthwaite Water is proposed, and the part played by these various groups of bacteria in creating zones of microbiological activity within the lake discussed. Particular reference is made to the formation of an anaerobic zone in the hypolimnion of the lake, which results in the growth of photosynthetic bacteria at various layers in the water profile and the growth of members of the sulphur bacteria on the surface mud.

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THE PROPERTIES AND CLASSIFICATION OF THE PREDOMINANT GRAM-NEGATIVE BACTERIA OCCURRING IN SOIL

By A. J. HOLDING
School of Agriculture, Edinburgh

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1. Introduction

A large amount of the work done on soil Gram-negative bacteria has been concerned with the ability of the organisms, usually isolated from enrichment cultures or on highly selective media, to carry out specific chemical changes. For example, information is available on types of Gram-negative bacteria which can decompose cellulose, xylan, chitin, pectic and fatty substances, proteins, hydrocarbons and certain herbicides and antibiotics. Similarly, Gram-negative bacteria which can bring about such processes as nitrification and denitrification or which can assimilate gaseous nitrogen have been examined. An estimation of the numbers of these bacteria in soil has not normally been made, but it is probable that they make up only a small fraction of the total Gram-negative bacterial population. Very few investigators have isolated and examined the predominant Gram-negative bacteria occurring in soil or shown in what proportion they occur in the total soil microbial population. The object of the present work was to obtain more information about these predominant types. The organisms were isolated from plant-free soil and the rhizosphere and from soil after certain organic materials had been added. Several properties of the bacteria were examined and an attempt was made to classify the organisms.

2. MATERIALS AND METHODS

Rhizosphere experiments. Oat, red clover and perennial ryegrass plants were used for most of the experiments; wheat, leek, cabbage, carrot, beetroot and French bean plants were used occasionally. The plants were grown either in the field or in sieved (2 mm) soil in pots in the open. A rhizosphere was examined by macerating the entire root system and the closely attached soil in a sterile mortar. Using sterile tap water the macerate was washed into a dilution bottle and the volume was made up to 100 ml. In the field experiments plant-free control soil was taken approximately 6–9 in. from the extremity of the root region. Unplanted soil was used as the control for the pot experiments. The control soil was obtained at the time of harvesting the plant; the first dilution was made with 10 g of soil and 90 ml of sterile tap water.

Experiments on the addition of organic materials. Sterile aqueous solutions of glucose, DL-malic acid, yeast extract (Difco), vitamin-free Casamino Acids (Difco) and peptone (Evans) were added separately to sieved soil contained in open Petri dishes. The materials were added to give a concentration of 0·2% (w/v) in the soil moisture which was maintained at approximately 70% of the water holding capacity by adding sterile tap water. Duplicate dishes were incubated at room temperature (18–20°) and examined after 2 and 7 days respectively. Fresh sieved soil and soil to which only sterile tap water had been added were used as controls. For an examination, the entire contents of the Petri dish were washed into a dilution bottle with sterile tap water and the total volume made up to 100 ml.

Total count of viable micro-organisms. Decimal dilutions were prepared in sterile tap water and plated in triplicate with soil extract agar (Cunningham, 1947, p. 40) to which 0·2% (w/v) of yeast extract (Difco) was added. The plates were incubated at 22° for 14 days before all the colonies were counted, including those of actinomycetes and fungi.

Isolation and count of Gram-negative bacteria. Holding (1954) found that of several media tested, nutrient agar containing 1:500,000 crystal violet (Gurr) gave the highest count of soil Gram-negative bacteria. The medium contained peptone, 5·0 g; meat extract (Lab-Lemco), 5·0 g; agar (Davis, New Zealand), 15·0 g; tap water, 1·0 l.; pH 6·8; sterilized momentarily at 22 lb/in^2. The sterile crystal violet was added aseptically immediately prior to pouring the agar. Counts were obtained by surface plating 0·1 ml of the diluted suspension and counting the colonies which developed after incubation at 27° for 7 days. Isolations were made by picking several representatives of each different colony type from plates showing between 30 and 300 colonies. The isolates were purified by plating on nutrient agar. Stock cultures were maintained on slopes of dilute Yeastrel-peptone agar (Topping, 1937) held at room temperature.

Biochemical and physiological tests

The Gram-negative bacteria were examined mainly for characteristics which might lead to a better understanding of factors which influence their development in soil.

General procedure. All the media were sterilized momentarily at 22 lb/in^2 except the milk, which was steamed. Inocula were obtained from 2–3 day slope cultures on Topping's medium. Cultures were incubated at 27° and, except where stated otherwise, examined daily.

Utilization of sugars. For the aerobic utilization of sugars the medium of Board & Holding (1960) (referred to hereafter as Medium A) was used. It contained: $NH_4H_2PO_4$, 0·5 g; K_2HPO_4, 0·5 g; yeast extract (Difco), 0·5 g; agar, 5·0 g; distilled water, 1·0 l.; bromothymol blue (B.D.H.); a mineral supplement; pH 7·2. The sugars were prepared as filter-sterilized aqueous solutions and were added to the sterilized Medium A (10 ml) before the agar solidified to give a final concentration of 0·5% (w/v). Stab inoculations were made and the cultures were examined for an acidic reaction which indicated that the sugar was being oxidized. With bacteria unable to oxidize the sugar, the reaction of the medium remained unchanged or became slightly more alkaline.

The examination of the anaerobic utilization of glucose was carried out by stab inoculating the cultures into 10 ml of nutrient agar to which had been added the indicator bromocresol purple (B.D.H.) and 0·5% (w/v) of glucose.

Utilization of malate and citrate. The two media were prepared by adding 15·0 g of agar and either Na citrate $2H_2O$, 5·0 g or DL-malic acid, 5·0 g along with the other constituents of Medium A before adjustment of the pH and sterilization. The development in the sloped culture (5 ml) of a more alkaline reaction than in Medium A to which no organic acid compound had been added indicated that the organic acid was being oxidized.

Utilization of amino acids. To Medium A from which the agar, yeast extract and bromothymol blue had been excluded, 5·0 g of Casamino Acids (Difco, not vitamin-free) were added. Bacteria were inoculated into 5 ml of this and other liquid media by adding a drop of a slightly turbid suspension in 0·85% (w/v) of NaCl. Visible turbidity was taken to indicate that the medium was suitable for the growth of the organism. Cultures showing only slight turbidity were reinoculated into another tube of the same medium to ensure that the growth was not due to materials carried over from the saline suspension.

Nitrogen requirements. Three liquid media were used to investigate the nitrogen sources which would support the growth of the bacteria. The basal nutritional medium (Medium B) contained only mineral nitrogen and had the following composition: glucose, 5·0 g; Na citrate $2H_2O$, 1·0 g; Na acetate $3H_2O$, 1·0 g; Na succinate $6H_2O$, 1·0 g; Ca gluconate H_2O, 1·0 g; $NH_4H_2PO_4$, 1·0 g; K_2HPO_4, 0·08 g; KH_2PO_4, 0·02 g; KNO_3, 1·0 g; a mineral supplement; distilled water, 1·0 l. The medium was autoclaved momentarily at 5 lb/in^2 and the precipitate filtered off before tubing and sterilization. In the preparation of the other two media an organic nitrogen source was added to Medium B. An amino acid-containing medium was prepared by adding 5·0 g of vitamin-free Casamino Acids (Difco) and the addition of 10% (v/v) of yeast autolysate (Cunningham, 1947, p. 42) provided a third medium which contained the more complex nitrogen compounds and growth factors.

Starch hydrolysis. The bacteria were spot-inoculated on to plates of nutrient agar containing 0·2% (w/v) of soluble starch. After incubation for 7 days the plates were flooded with dilute iodine.

Xylan and cellulose hydrolysis. The two media were prepared by adding to Medium A (containing 15·0 g of agar) 5·0 g of partially purified xylan and 5·0 g of precipitated cellulose (kindly donated by Dr. G. O. Aspinall and Miss P. Dunn respectively). The plates were incubated for 6 weeks in a moist atmosphere and examined for the development of a clear zone round the colony.

Pectin breakdown. A gel was prepared according to the method of Wieringa (1947) using sodium pectate (A.S.P.). Yeast extract, 0·5% (w/v), was added to Wieringa's agar medium and incubation was for 14 days.

Proteolytic action in milk. Cultures in skimmed milk were examined after 1, 2 and 6 weeks.

Position of flagella. The methods of Gray (1926) and Rhodes (1958) were used for staining the flagella of bacteria grown for 18–24 hr on Topping's medium.

3. CLASSIFICATION

Most of the characteristics upon which the classification was based are included in Table 1. Action on glucose, nitrogen compounds required for growth, pigment production, type of motility, and position of flagella, if present, have been the main criteria used. Several characteristics appeared to have no differential value. All the organisms oxidized amino acids and malate; the majority oxidized citrate. Of the sugars employed, glucose probably gave all the significant information. Rate of growth, colonial morphology, intensity of pigmentation and certain other properties varied within the groups into which the bacteria were placed. The bacteria were rod-shaped except where otherwise stated. These variable characteristics could not be correlated with any other property of the organisms.

Table 1. *Some properties of the predominant Gram-negative bacteria of the soil*

Group	Oxidation‡ of								Growth‡ in nutritional media containing			Position of flagella
	Glucose	Fructose	Xylose	Maltose	Sucrose	Malate	Citrate	Amino acids	Mineral nitrogen	Amino acids	Yeast extract	
Pseudomonas	+	+	+	(+)	(+)	+	(+)	+	+	+	+	Polar
Pseudomonas A	+	+	(−)	+	(+)	+	+	+	−	(+)	+	
Pseudomonas B	−	−	−	−	−	+	(+)	+	(+)	(+)	+	
Xanthomonas	+	+	(+)	+	+	+	(+)	+	−	(+)	+	
Chromobacterium	+	*	*	*	*	*	*	+	+	+	+	
Agrobacterium	+	+	+	+	+	+	+	+	+	+	+	Peritrichous
Flavobacterium	+	(+)	+	+	+	+	+	+	−	(+)	+	
Achromobacter†	+	+	(+)	+	+	+	(+)	+	−	(+)	+	
Alcaligenes	−	−	−	−	−	+	+	+	(+)	+	+	
Aeromonas	F	+	+	+	+	+	+	+	+	+	+	Polar
Aerobacter	F	+	+	+	+	+	+	+	+	+	+	Peritrichous
Bacterium herbicola	F	+	+	+	+	+	+	+	+	+	+	
Cytophaga	+	+	+	+	+	+	(−)	+	(+)	(+)	+	

‡ Oxidation or growth: +, all strains positive; −, all strains negative; (+), majority of strains positive; (−), majority of strains negative; F, fermentation (only glucose examined). * Examination not carried out.
† Nonmotile strains were occasionally isolated.

Aerobic bacteria possessing polar flagella

Pseudomonas–Pseudomonas A–*Pseudomonas* B *groups.* Strains which would grow in Medium B and oxidize glucose were identified as *Pseudomonas*. A large proportion of the strains produced a fluorescent pigment easily visible under ultraviolet light.

Some however did not produce a pigment even in the medium of Paton (1959). The bacteria placed in the *Pseudomonas* A group oxidized glucose but required an organic nitrogen source usually in the form of amino acids. The bacteria placed in the *Pseudomonas* B group were apparently unable to oxidize carbohydrates. Neither of the latter two groups produced a fluorescent pigment.

Xanthomonas. The yellow pigmented bacteria isolated in this study differed from the plant pathogenic species of *Xanthomonas* by not producing copious slime on sugar-containing media, and on repeated subculture many strains lost their ability to produce pigment. The tests used in this study did not differentiate the *Pseudomonas* A group from the *Xanthomonas* strains which had lost their ability to produce the yellow pigment.

Chromobacterium. Bacteria producing a violet pigment were placed in this genus.

Aerobic bacteria possessing peritrichous flagella

The morphology of the bacteria varied from cocci and coccobacilli to rods of variable size. The number of flagella, which was variable, could not be correlated with any other characteristics.

Agrobacterium. Unpigmented bacteria capable of oxidizing glucose and of growth with only a mineral nitrogen source were considered to be strains of *Agrobacterium*. Bacteria with the properties of the closely related genus *Rhizobium* were not isolated.

Flavobacterium. Yellow pigmented bacteria with the ability to oxidize glucose were placed in this genus. The pigment is normally a deeper shade of yellow than that found in the *Xanthomonas* group.

Achromobacter. Unpigmented bacteria capable of oxidizing glucose but requiring an organic nitrogen source were allocated to this genus. Nonmotile strains, which were isolated only occasionally, occurred as a very small proportion of the Gram-negative population.

Alcaligenes. Unpigmented and yellow bacteria unable to oxidize glucose were assigned to this genus.

The facultative anaerobes

All the strains encountered gave a positive Voges-Proskauer reaction and appeared to have the type of glucose fermentation associated with the Aerobacter group.

Aeromonas. Unpigmented bacteria possessing polar flagella were placed in *Aeromonas*. Some strains did not produce gas from glucose.

Aerobacter. Unpigmented bacteria possessing peritrichous flagella were placed in this genus. A large proportion of the strains did not produce gas from glucose.

Bacterium herbicola. The yellow pigmented bacteria were identified as *Bacterium herbicola*, whose properties have been reviewed by Mack (1936). The bacteria isolated in this study possessed peritrichous flagella and could be regarded as members of the *Enterobacteriaceae*.

Cytophagas

The demonstration of gliding motility remains the only reliable method of identifying these organisms. The motility was usually more readily observed on media containing low concentrations of nutrient materials. Some strains which showed indefinite gliding motility were included in this group.

4. OCCURRENCE

(a) *In plant-free soil and in the rhizosphere*

Data obtained in studying 26 samples each of plant-free soil and the rhizosphere are shown in Table 2. The composition of the rhizosphere microflora was not clearly related to the type of plant, the time of harvesting or to growth in the field or in pots.

The wide variation in the proportion of Gram-negative bacteria in the soil microflora and the preferential promotion of their growth in the rhizosphere has been reported by many previous workers, including Lochhead (1940), Clark (1940) and King & Wallace (1956). The results obtained in this study indicate that the predominant types in plant-free soil frequently occurred as predominant types in the rhizosphere. The numerical importance of *Pseudomonas* strains in both environments was clearly shown. Besides being the only group to be isolated from all the samples they occurred usually in a much larger proportion than the other groups. It is of interest to note, however, that whilst their proportion in the total microflora increased from about 3·5% in the plant-free soil to about 10% in the rhizosphere, their proportion in the

Table 2. *The occurrence of Gram-negative bacteria in plant-free control soil and the rhizosphere*

Group	Plant-free soil*			Rhizosphere*		
	No. of samples from which isolated	Variation in proportion†	Average proportion†	No. of samples from which isolated	Variation in proportion†	Average proportion†
Total Gram-negative organisms	26	2–32	7	26	7–41	20
Pseudomonas	26	4–79	48	26	15–84	51
Pseudomonas A	17	6–25	11	7	8–49	6
Pseudomonas B	15	10–47	20	11	7–65	11
Xanthomonas	8	6–19	4	15	2–25	6
Chromobacterium	3	20–40	4	2	10–16	1
Agrobacterium	0	—	—	6	18–35	7
Flavobacterium	0	—	—	1	8	<1
Achromobacter	10	9–32	7	11	8–36	4
Alcaligenes	0	—	—	9	4–65	5
Aeromonas	0	—	—	3	8–11	1
Aerobacter	0	—	—	2	1–24	1
Bacterium herbicola	0	—	—	1	1	<1
Cytophaga	15	2–29	6	13	2–24	6

* Twenty-six samples of each were examined. † The total Gram-negative bacteria are expressed as percentages of the total microbial flora; the Gram-negative groups are expressed as percentages of the total Gram-negative flora.

Gram-negative bacterial population only increased to a slight extent. In similar studies Clark (1940) found that the proportion of *Pseudomonas* strains in the rhizosphere of some types of plant was greatly increased compared with the plant-free soil. But whereas Gyllenberg (1955) found that a large proportion of the Gram-negative bacteria which she isolated from the rhizosphere of graminaceous plants belonged to the genus *Pseudomonas*, Sperber & Rovira (1959) concluded that a large proportion of the rhizosphere Gram-negative bacteria which they isolated belonged to the genus *Arthrobacter*. The development of *Pseudomonas* A and B in the rhizosphere is difficult to interpret since they were detected less frequently but sometimes in a higher proportion than in the plant-free soil. Strains of *Cytophaga* occurred unexpectedly among the predominant Gram-negative soil organisms. It is apparent that conditions in soil suitable for their multiplication were not different from those required by the other bacteria isolated in this study. Previously this group has been associated only with the breakdown of complex materials such as chitin and cellulose. Strains of *Xanthomonas* occurred fairly frequently in both environments, but it was of interest that strains with the characteristics of the plant pathogenic species were not isolated.

Certain groups which were not isolated from the plant-free soil were apparently favoured in some rhizospheres. No characteristics which they were found to possess in common seemed to account for their development. Whereas the *Alcaligenes* group and facultative anaerobes have rarely been detected in the rhizosphere by previous workers, much attention (e.g. Starkey, 1929*a*,*b*) has been given to organisms that form mucoid colonies, which were often assumed to belong to *Agrobacterium*. The results obtained in this study indicate that strains of *Agrobacterium* could make up a fairly large proportion of the Gram-negative bacterial flora of the rhizosphere, but that their development was very irregular.

(b) *In soil after the addition of certain organic materials*

The results of the experiment are presented in Table 3. *Pseudomonas* strains were the most important group numerically and they responded to the large amount of available nutrient materials more than the other predominant groups in the untreated soil. These other groups often occurred as predominant types in the treated soils, but always in smaller proportions than in the untreated soils. In general, all the treatments appeared to have a similar effect on the composition of the Gram-negative bacterial population. The differences which did exist could not be correlated with the properties of the added material. The changes after the addition of tap water were attributed to the increased availability of soluble nutrient materials already present in the soil. Similarities between these results and those obtained in the rhizosphere studies suggest that available energy sources at the root surface could be promoting the development of the predominant Gram-negative population.

Although all the added materials increased the numbers of micro-organisms in the soil, the largest total count developed after the addition of yeast extract, while the Casamino Acids and peptone additions promoted the growth of the highest numbers and largest proportions of Gram-negative bacteria. One interpretation of these results is that in this soil, when energy sources were available, lack of factors found in yeast extract limited the multiplication of Gram-positive and Gram-variable bacteria while shortage of available nitrogen sources limited the Gram-negative types.

Table 3. *The effect of adding organic materials to soil on the Gram-negative bacteria*

	Numbers* or proportions† of bacteria in soil treated with						
	No addition‡	Tap water	Glucose	Malic acid	Yeast extract	Peptone	Casamino Acids
Total count(10^6/g)	34	34·5	107	102	270	158	153
Count of Gram-negative bacteria (10^6/g)	1·1	4·1	21	33	43	55	80
% Gram-negative bacteria	3	12	20	32	16	35	52
Pseudomonas	45	72	57	70	79	86	90
Pseudomonas A	18	9	5	9	0	0	5
Pseudomonas B	0	0	0	6	0	5	0
Xanthomonas	0	3	0	0	0	0	0
Agrobacterium	0	0	0	0	0	5	0
Flavobacterium	0	1	5	0	0	0	0
Achromobacter	0	0	23	0	5	0	0
Alcaligenes	22	8	10	12	14	2	5
Cytophaga	15	7	0	3	2	2	0

* Averages of results, which were similar, obtained after incubation at 18–20° for 2 and 7 days.

† The results for the Gram-negative groups are expressed as percentages of the total Gram-negative flora.

‡ Fresh soil examined immediately after sampling.

5. Nutritional Requirements

An examination of certain nutritional requirements was carried out in an attempt to elucidate some of the factors influencing the development of the Gram-negative bacteria.

Table 4. *Nutritional grouping of Gram-negative bacteria*

Nutritional group	Results* for organisms isolated from				Results* for organisms isolated from soil supplemented with						
	Plant-free soil		Rhizosphere								
	Variation in proportion	Average proportion	Variation in proportion	Average proportion	No addition†	Tap water	Glucose	Malic acid	Yeast extract	Peptone	Casamino Acids
Grow with mineral nitrogen	10–100	63	30–100	71	58	75	81	70	81	93	90
Require amino acids	15–72	23	0–66	16	12	10	5	9	0	0	5
Require yeast extract	0–65	14	0–52	13	30	15	14	21	19	7	5

* Results expressed as percentages of the total Gram-negative flora.

† Fresh soil examined immediately after sampling.

A large variation in the proportions of the nutritional groups in the samples of plant-free soil and rhizosphere is shown by the results presented in Table 4. The increase in the rhizosphere of the proportion of bacteria requiring only a mineral nitrogen source was interesting since studies with the total bacterial flora (Lochhead & Rouatt, 1955; Gyllenberg, 1957) have shown that there was an increase in the proportion of organisms requiring amino acids. The methods employed do not appear to account for the difference.

The addition to soil of the organic materials and tap water brought about large increases in the proportion of bacteria requiring only a mineral nitrogen source. The increases were attributable mainly to strains of *Pseudomonas*. The results obtained after the addition of Casamino Acids, peptone and yeast extract were particularly interesting, since there was not the preferential promotion of the growth of bacteria requiring the nitrogen compounds which these materials supply. All the results were similar to those obtained in the rhizosphere studies. It seems likely therefore that the effect of plant growth on the nutritional groups of bacteria could be due solely to the liberation by the root of any materials similar to those used in this study.

6. ACTION ON COMPLEX ORGANIC COMPOUNDS

The Gram-negative bacteria isolated from 6 samples of plant-free soil and a rhizosphere in each of the same soils were examined for their ability to utilize certain complex organic compounds.

Table 5. *The ability of isolates from plant-free soils and rhizospheres to decompose certain complex organic compounds*

Group	Decomposition* of				
	Casein	Pectin	Starch	Xylan	Cellulose
Pseudomonas	±	(−)†	−	−	−
Pseudomonas A	±	−	−	−	−
Pseudomonas B	−	−	−	−	−
Xanthomonas	±	−	−	−	−
Chromobacterium	+	−	−	−	−
Agrobacterium	−	−	−	−	−
Flavobacterium	−	−	−	−	−
Achromobacter	−	−	−	−	−
Alcaligenes	−	−	−	−	−
Aeromonas	+	−	(+)	−	−
Aerobacter	−	−	−	−	−
Bacterium herbicola	−	−	−	−	−
Cytophaga	±	−	(+)	−	−

* Decomposition: +, all strains positive; −, all strains negative; ±, positive and negative strains in approximately equal proportions; (±), majority of strains positive; (−), majority of strains negative.

† Positive results were obtained only with rhizosphere isolates; all the soil isolates were negative.

A feature of the results (Table 5) was the apparent inability of a very large proportion of the bacteria to attack any of the complex carbohydrates examined. Major plant constituents such as cellulose and xylan, which were not attacked by any of the organisms, are unlikely therefore to be directly concerned with the development of the predominant Gram-negative population in soil. Most strains of *Cytophaga* hydrolyzed starch, but the results shown in Table 3 indicate that there are other substances which could promote the growth of these bacteria in soil. Pectate gel was liquefied by an average of 37% of the *Pseudomonas* strains isolated from the rhizospheres, the proportion varying from 0–60%. This was the only instance of a characteristic being possessed by rhizosphere bacteria but not by the plant-free soil isolates. Protein breakdown, as indicated by casein hydrolysis, was brought about by between 30–40% of all the isolates, although the proportion in the different samples was very variable. There was no preferential promotion of the growth of proteolytic types in the rhizosphere. It seems unlikely that the ability of the bacteria to attack the complex organic compounds is associated directly with their development in the rhizosphere.

7. SUMMARY

The Gram-negative microflora of untreated plant-free soil was compared with that of the rhizosphere and of soil treated with certain readily available organic materials. The average proportion of Gram-negative bacteria in 26 soil samples was 7%, in the rhizospheres 20%, and much larger proportions were found after the addition of some of the organic materials. Fluorescent pseudomonads and closely related nonfluorescent types usually made up a large proportion of the Gram-negative populations. Other types of *Pseudomonas* and strains of *Cytophaga*, *Achromobacter*, *Xanthomonas*, *Chromobacterium*, *Alcaligenes*, *Agrobacterium*, *Flavobacterium* and the facultative anaerobes were also numerically important in certain samples. The dominant types in the untreated soil frequently occurred as dominant types in the rhizosphere and treated soil. An examination of the nutritional requirements of the bacteria showed that, compared with the untreated soil, there was in the rhizosphere and in treated soil an increase in the proportion of bacteria requiring only a mineral nitrogen source. Similarities between the rhizosphere and treated soil populations indicated that various organic materials liberated at the root surface could be promoting the development of the rhizosphere population. The activity of the bacteria on certain complex organic compounds could not account for their development in the rhizosphere.

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(Symposium on *Pseudomonas* and *Achromobacter*: Paper XI)

THE ROLE OF *PSEUDOMONAS* IN PLANT DISEASE

By A. M. PATON

Department of Bacteriology, University of Aberdeen

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1. Introduction

In the time scheduled for this contribution to the Symposium it would be unprofitable, as well as presumptuous on my part, if I were merely to reiterate the reviews of Kern (1956), Wood (1955), Brian (1955), Barnett (1959) and Starr (1959), which in recent years have done so much to consolidate our knowledge of the plant diseases caused by bacteria.

Moreover, I have released myself from the necessity of discussing a series of particular diseases merely to illustrate the already well known fact that pathogenic conditions brought about by pseudomonads are both common and of great economic importance. Instead I wish to point out a few items of outstanding interest in the literature of the last few years and to supplement them with some recent observations.

2. Classification

Much of the energy which has been expended on research into the activities of organisms of the genus *Pseudomonas* has been directed towards the elucidation of fundamental problems concerning metabolic pathways; but information as to the general applicability and usefulness of such findings is often slow in reaching the applied bacteriologist.

The bacteriologists concerned with plants have so often in the past limited their attentions largely to the isolation and identification of organisms. As a result we have, in plant pathology, a vast accumulation of names of organisms and their hosts which is, for the most part, only of limited value.

This generally unsatisfactory situation has been made even less acceptable by the attention drawn in recent works by Fuchs (1957), Dye (1958) and Starr (1959) to the fallacy of naming species according to the host plants on which the organisms are commonly found. Fuchs of Holland, for example, showed that a number of organisms pathogenic to plants and previously considered as distinct species within the genus *Pseudomonas* could be conveniently and naturally grouped together on the grounds of host affinities and biochemical characters. Valuable additional support for a reconsideration of organisms of specific rank comes from Dye of New Zealand, who found in his work in Edinburgh on the closely related genus *Xanthomonas* (Dye, 1958) that many of these pathogens were by no means host specific and that organisms isolated from a wide variety of plants could, after short introductory plant passages, infect a common host. If this situation is admitted, as indeed it must, considerable effort must be made towards a redefinition of these bacteria into groups which are of practical value both to the taxonomist and to the pathologist. It is probable, however, that the information on which a satisfactory alternative grouping could be based does not yet exist and we must await the outcome of work in the relatively unexplored field of host-pathogen relationships—not forgetting commensalism, about which our ignorance is profound!

3. DISTRIBUTION

Representatives of the genus are to be found almost universally in nature. This is not altogether unexpected as most of them are capable of active growth in the presence of simple nutrients. There the simplicity ends, for they are equipped with enzyme systems capable of breaking down a wide range of complex organic compounds and releasing as metabolic products an even wider variety of substances than is generally realized.

Although the pseudomonads are of widespread occurrence, their numbers do not appear to be higher than those of related genera in many natural environments. An exception is the normal plant environment, where pseudomonads apparently find a very congenial habitat. This becomes very evident to the bacteriologist who attempts the isolation of a pathogenic strain from a diseased plant, when a number of different pseudomonads may be isolated in addition to the pathogen. The isolation of the pathogen is commonly a matter of trial and error, as the cultural and biochemical characters of the types isolated may be very similar. This similarity, confusing as it is to the pathologist, may provide some interesting information on host-pathogen relationships.

4. LEVAN PRODUCTION

There is evidence to support the belief that a large proportion of the pseudomonads associated with plant tissue are of a group characterized by the ability to produce levan polysaccharide from sucrose (Paton, 1956). The synthesis of levan was once thought to be mainly restricted to certain pathogens, but it is now known that many members of the saprophytic pseudomonad population of plants have this property. This does not preclude the finding of levan forming types of this genus associated with soil and water. The occurrence of such organisms apart from plants is emphasized in Fuch's (1959) comprehensive account of levan formation by bacteria. Mention was made in a paper to this Symposium (Klinge, 1960) of the occurrence of levan producing pseudomonads in blood transfusion bottles. Whether or not these organisms have been removed from their normal habitat of plant surfaces is not yet clear.

It appears that the pseudomonads are closely associated with the plant surface and are not readily removed. This can be effectively demonstrated in the case of the washed roots of young, naturally grown plants such as clover and oats, where impression films of the finer roots show a sheath of Gram-negative rods which can be identified further under the fluorescence microscope using labelled antiserum (Chadwick, McEntegart & Nairn, 1958) specific for this levan producing, rhizoplane *Pseudomonas*—a common plant saprophyte (see Plate 1, a).

The pathogenic organisms, during their passive periods on plant tissue, must presumably live and multiply, as do the associated saprophytes, in an environment where nutrients are not constantly or readily available. The ability of most members of both groups to produce polysaccharide may not be entirely without reason. It has been suggested that polysaccharide as a capsule, or in more diffuse form, serves to protect the cells from desiccation and from bacteriophage attack; but it is possible that the function is of greater fundamental value to the organisms in this particular environment. It is a well known fact that the formation of polysaccharide from simple sugars creates an osmotic gradient which tends to attract solutes towards the polymer until an equilibrium is reached. That something of this nature occurs to an effective degree in the case of bacteria can be readily demonstrated by growing the organisms on sucrose agar containing aniline blue. The polysaccharide producing colonies absorb the dye whereas the others remain colourless. The intensity of the attraction can be judged by a channel plate technique (see Plate 1, b and Fig. 1). If this is related to the plant organisms it can be recognized as a possible method of drawing nutrients towards and concentrating them in the immediate environment of the microbial cells. It is realized that only micro-quantities of polysaccharide

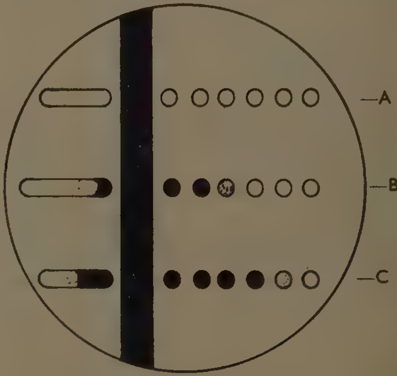

Fig. 1. Diagrammatic representation of the channel plate technique. A channel is cut out of a plate of nutrient agar containing 5% (w/v) of sucrose and replaced with the same medium plus 0·1% (w/v) of aniline blue.
Levan production as shown by absorption of dye: A, negative; B, positive; C, strongly positive.

may be produced from the restricted resources available on, say, leaf surfaces, but the general effect on the survival and multiplication of the organisms would be profound. Levan may not necessarily be the only or even the main polysaccharide involved. More extensive information on the nature of normal leaf excretions would help to elucidate this.

5. INVASIVE ABILITY

The plant pathogens, however, are not content to live passively on the surface of plant tissue but take every opportunity of invading their hosts. By diverse means, but usually through natural apertures or wounds, to circumvent the cutinized and otherwise protected surfaces, the bacteria become intimately associated with the relatively undefended inner cellular structure. The ability to invade further into the tissue at a rate greater than the host can control is an essential character of a virulent pathogen. *Pseudomonas* spp. are no exceptions—a fact realized only too well by agriculturists all over the world.

The basis of pathogenicity remains to a very great extent an unsolved mystery. For many years now observations have been made on the formation of phytotoxic materials which could be made to reproduce the typical symptoms of a number of bacterial plant diseases. For example, a number of polysaccharide containing materials have been isolated from bacterial cultures or infected plants which were shown to induce wilting effects. But, as Starr (1959) has emphasized recently, this apparent toxicity may be due to mechanical interference with the transpiration system of the host. It does seem more than a coincidence, however, that an increase in the ability to produce slime was found by Corey & Starr (1957) to be paralleled by an increase in the virulence of mutants of at least one plant pathogen.

Most recent reviewers of this subject have noted with interest that chlorotic haloes similar to those produced in tobacco wildfire disease (caused by *Ps. tabaci*) can be induced by a derivative of a diaminodicarboxylic acid present in culture filtrates of the pathogen. It is authoritatively suggested (Woolley *et al.* 1952) that the toxin interferes with the methionine metabolism of the plant. Although the presence of substances having similar gross effects on plant tissue has been demonstrated in other diseases, there is, as yet, no evidence that the active agent is the same. The finding of this *Ps. tabaci* toxin is undoubtedly of outstanding interest, but the presence of such material among the metabolic products of an organism does not provide sufficient evidence to brand it as a pathogen. The formation of toxic material would, no doubt, assist the establishment of an organism within plant tissue by either killing the surrounding cells or by inhibiting their reactivity against the invading bacteria; but this factor by itself does not account for the usual rapid spread of the disease.

6. SPECIAL PROPERTIES OF PLANT PATHOGENS

The qualities which together make certain bacteria aggressive towards plant tissue are largely unknown, but would, conceivably, include the ability to: (1) live actively on the nutrients present in the host tissue; (2) live actively in the specific pH and eH environment existing in the normal plant tissue, or adapt that environment to one more suitable; (3) produce a toxic substance such as is formed by the tobacco pathogen; (4) spread pathogenic effects through the tissue beyond the areas of actual infection and thus prepare the way for further invasion. This last mentioned ability has stimulated both interest and speculation, but there is no direct evidence that it is due to any specific factor produced by the bacteria.

The common observation of the remarkable rate of spread of pathogenic conditions within susceptible plants has led to the suggestion that a spreading factor analogous to the hyaluronidase of animal pathogens may be involved. The likelihood of such a substance, enzymic or otherwise, being formed can be rated high; but the difficulty is in proving its existence and in demonstrating its effect. Kern (1956), in his admirable review, provides some circumstantial evidence in support of a spreading factor, but little other information is available other than the suggestions that the substance involved may be either enzymatic, like the cellulolytic enzymes, or nonenzymatic, like trimethylamine. It would not be surprising to find that the pectin complexes associated with plant cells are intimately concerned in any change likely to promote the spreading of the bacterial metabolites.

It is well known that numerous plant pathogens are capable of causing the ultimate soft rot of their hosts, but at least as many others, being devoid of the necessary enzyme systems, cannot bring this about. We find both groups within the genus *Pseudomonas*. As with soft rotting organisms of other genera, polygalacturonases (pectin glycosidases) are produced. Pectin methylesterase is found rarely, if at all (Smith, 1958).

A comparison of the activities of the two groups of pseudmonads reveals some interesting information. If pathogenic soft-rotting types (e.g. certain strains of *Ps. syringae*) are grown on nutrient agar plates within which are embedded fresh, sterile slices of plant tissue such as potato tuber, turnip, or cucumber, the resulting dissociated cells are killed as judged by the neutral red plasmolysis technique (Plate 1, c and Fig. 2) and here the work of Tribe (1955), Lapwood (1957) and Fushtey (1957) must be considered relevant. That this should occur is perhaps not surprising, but if rotting is inhibited by lowering the pH of the agar well below the optimum of the polygalacturonases (e.g. to pH 4·5) the cells of the embedded tissue can again be shown to die under the influence of the pathogen. A slow or partial activity of polygalacturonase might then be suspected, but this can be ruled out because other

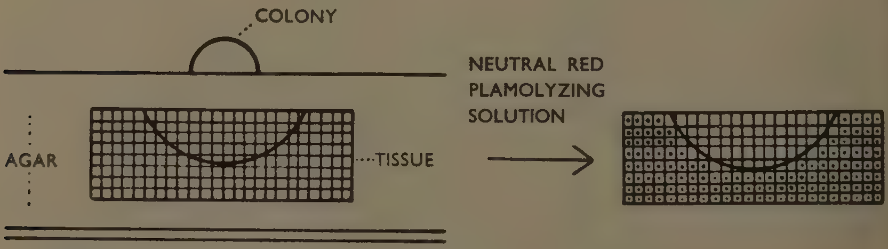

Fig. 2. Tissue embedding technique.
Right hand drawing: dotted squares, normal plasmolysis; plain squares, no plasmolysis.

virulent *Ps. syringae* strains which do not produce polygalacturonase show an identical effect. The death of the plant cells under these conditions is often associated with a clearing of the tissue—a phenomenon best demonstrated in the case of unripe cucumber slices where there is a noticeably increased transparency of the cell walls without loss of rigidity (Plate 1, d). It is not certain whether both these phenomena are manifestations of the same or different activities, but these observations show that at least some pathogenic pseudomonads can affect plant cells at some considerable distance from the area of actual infection without the aid of known pectolytic enzymes, even if these are produced. This technique can be used to compare the effect of bacteria on a variety of sterile plant tissues. The influence of different chemicals on pathogenicity can be examined by altering the composition of the agar medium surrounding the plant section.

It may be interesting to note at this stage that the pretreatment of plant tissues such as potato tuber and turnip root with dilute solutions of the powerful calcium chelator ethylenediaminetetra-acetic acid (EDTA), greatly increases the rate of cell disintegration by soft-rotting organisms and, moreover, it is possible to isolate pseudomonads from the soil which, although they possess polygalacturonase activity, are unable to rot the usually susceptible tissue unless it is treated with EDTA.

The question of the existence of a more complex protopectin or 'native pectin' in the normal plant matrix has been the subject of discussion for many years, and opinion is still sharply divided over the issue. In a recent review by Demain & Phaff (1957) the possibility of the occurrence of a 'non-pectolytic macerating enzyme' was not ruled out. Much of the evidence against such a substance being even necessary is, however, based on work with crude enzyme preparations from fungi.

7. SPECULATION

Obviously such simple observations are by no means sufficient in scope and in detail to provide more than a few pointers for future investigators, but it might be reasonable to consider how a spreading factor might fit into our present knowledge of plant infection from the stage of the initial inoculation with a virulent pathogen. The organism at the point of entry into the tissue will be bathed in the nutrients or the inter- or intracellular fluids. Although at a suboptimal pH, it is likely that some growth will take place at the expense of the nutrients immediately available. The cellular organisation of the plant tissue will render immediate further supplies of nutrients only slowly available; and at this early state the pH of the environment will still be too low to promote a marked pectolytic activity (optimum *c.* pH 7). If a substance or substances which can make the pectin more permeable and also kill the surrounding cells now diffuses from the organism, a considerable supply of nutrient would be released to the bacteria. The rapid change of pH towards alkalinity which would occur at this stage might provide more suitable conditions for pectin glycosidase and/or other activities deleterious to the plant.

The role of *Pseudomonas* in plant disease, although a major one, at present defies accurate definition; but at the same time it provides opportunity for fruitful research. This paper, intentionally discursive, may emphasize such opportunities.

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EXPLANATION OF PLATE

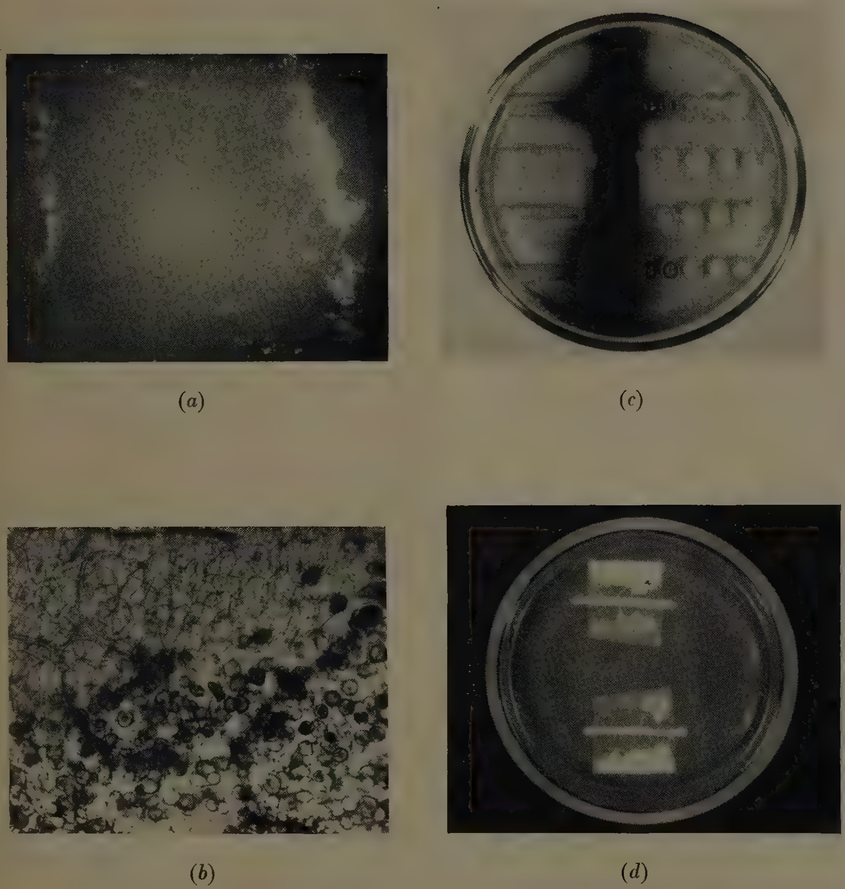
(a) Clover root impression film (× 75) showing appearance under the fluorescence microscope of a mass population of *Pseudomonas* on the root surface, stained specifically for the bacteria with antiserum conjugated with lissamine rhodamine B 200.

(b) Section of cucumber tissue (× *c.* 340), after application of the embedding technique, showing living and dead cells as demonstrated by plasmolysis in M KNO_3 containing neutral red. The dark lines are traces of vascular tissue.

(c) Channel plate technique showing diffusion of aniline blue towards and into the polysaccharide-containing colonies (cf. Fig. 1).

(d) Cucumber slices embedded in peptone agar showing 'clearing' caused by strains of *Ps. syringae*.

(*Received* 17 *October*, 1960)



(*a*)

(*b*)

(*c*)

(*d*)

PATON, A. M. — THE ROLE OF *PSEUDOMONAS* IN PLANT DISEASE. PLATE 1

Rogers, K. B. (1960). *J. appl. Bact.* **23** (3), 533–537.

(Symposium on *Pseudomonas* and *Achromobacter*: Paper XII)

PSEUDOMONAS INFECTIONS IN A CHILDREN'S HOSPITAL

By K. B. ROGERS
The Children's Hospital, Birmingham

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1. Introduction

At the present time most patients can be cured if they are admitted to hospital with a bacterial infection which has been contracted in the home; but it is unfortunate that in modern hospitals patients frequently become infected after they have been admitted and many of the infections so contracted are extremely difficult to treat. Paradoxically a hospital which is able to demonstrate episodes of cross infection is potentially safer than one for which it is claimed that cross infection never occurs, because the logical sequel to the disclosure of cross infection by an efficient bacteriological service is the evolution of effective preventive methods. Therefore, although I will describe cross infection epidemics in my own hospital, I would emphasize that each such episode has been a stimulus to discover a remedy which will prevent a repetition of that type of epidemic. The hospital where I work only admits children and unlike the hospitals for adults, where staphylococci are now creating a serious problem, our most dangerous cross infections are due to Gram-negative bacteria. For many years we had epidemics due to the enteropathogenic specific serological types of *Escherichia coli* (Young & Rogers, 1954) which cause infantile gastroenteritis: but we have evolved relatively effective methods of controlling this disease and now *Pseudomonas pyocyanea* has become our most dangerous cross infecting pathogen.

2. The Importance of *Pseudomonas pyocyanea*

Ps. pyocyanea is particularly dangerous because few of the modern antiseptics kill it: some equipment and apparatus cannot be sterilized by any method which employs heat, and when an attempt is made to effect sterilization by antiseptics it is not unusual for everything except *Ps. pyocyanea* to be killed. Once a child has become infected from such contaminated apparatus the situation immediately becomes worse, as this patient acts as an overflowing reservoir from which other patients and apparatus can be infected.

It is very important for a hospital clinical laboratory to identify *Ps. pyocyanea* rapidly; if the presence of *Ps. pyocyanea* is not observed and the treatment of the patient started early, especially if the affected patients are neonates or infants, the infection is liable to become generalized. It is then not easily controlled, except by the systemic administration of Polymyxin B, and this is an antibiotic which is not employed routinely. It is a sad outcome of the inefficiency of a tardy bacteriological laboratory if the identification of *Ps. pyocyanea* is so delayed that the organisms cause an overwhelming infection and the diagnosis is only made at an autopsy when the characteristic macroscopic and microscopic appearances become evident.

3. MODES OF INFECTION

At the Children's Hospital, Birmingham, most of our *Ps. pyocyanea* infections are nosocomial. One epidemic was due to the use of a polythene tube which was 'sterilized' with chlorhexidine. During operations on babies with congenital oesophageal atresia, a soft rubber catheter was employed to clear the trachea if mucus had to be removed, and after use this catheter was placed in the polythene tube referred to above, where it remained until the operation was completed. Over a period of six months five new born babies were infected in this way, but it was not until the fourth baby became infected that the existence of an epidemic was appreciated because the time between each infection was long, because each of the first three babies was nursed in a different ward, and because two of these three infants also had other congenital lesions. This is shown in Table 1. The last two of the five babies involved in this outbreak recovered when they were treated with Polymyxin B, and the epidemic was quickly controlled when Pyrex tubing was used in place of the plastic tube.

Table 1. *A summary of the cases in an epidemic of* Ps. pyocyanea *infections transmitted to new born babies from a contaminated tube*

Date of birth	Ward	Site infected	Infected*	Died*	Associated lesions found at autopsy
March 10	C	Oesophagus, bronchus	7	9	Genito-urinary abnormalities
May 7	A	Lung	7	7	None except pneumonia
May 27	B	Pharynx	3	4	Bladder neck obstruction; subdural haemorrhage; bilateral tentorial tears
July 5	A	Nose, eye	4 5	†	None
August 24	A	Lung abscess	5	†	None

* Days after admission.

† These patients recovered following treatment with Polymyxin B.

More recently children have been infected from electric suction machines in which chloroxylenol was employed to sterilize the aspirated material. Large numbers of *Ps. pyocyanea* were found to be present in the bottles attached to these machines, and we have not found the ideal antiseptic to employ in them: but regular sterilization by heat has reduced this hazard. We have also found that, just as with Cetrimide solutions, the stock bottles of chloroxylenol must be protected from contamination. Through an unfortunate change in routine, cork stoppers were being used in these stock bottles, and these corks were found to be heavily infected with both *E. coli* and *Ps. pyocyanea*. Cork stoppers have been condemned by many workers, notably Anderson & Keynes (1958), and the problem of preventing the contamination of antiseptic solutions was fully discussed in a leading article (Leader, 1958) of the British Medical Journal.

4. CASE HISTORIES OF SEPTICAEMIA DUE TO *PSEUDOMONAS* SPP.

A case of Ps. pyocyanea *infection.* A three month old girl attended the Casualty Department with an abscess under her chin: this was incised and cultures of the pus provided a pure culture of a penicillin sensitive *Staphylococcus aureus*. The abscess resolved well until it was treated with a Eusol dressing held in place by 'plaster', under which small pustules appeared. Thirteen days after the original attendance the child was admitted to the hospital because the pustules were occupying a large area of the chin and the centre of each appeared to be gangrenous. On admission the child was not clinically ill: the cultures of the pustules gave very heavy growths of *Ps. pyocyanea* and a few faecal type 'viridans' streptococci, but no staphylococci, even by the use of techniques which are designed to show small numbers of Gram-positive organisms which might be present at the same time as many Gram-negative bacteria (Rogers & Heslop, 1948). A nasal swab taken three days after admission yielded a heavy growth of *Ps. pyocyanea* and 0·125% Polymyxin cream was applied to the affected areas. By the following day the lesions had spread from the chin and involved the adjoining cheek, with very marked induration of the whole of the affected area, and on that day the child had a convulsion. Accordingly, a lumbar puncture was performed. This yielded a clear cerebro-spinal fluid, which had less than 1 cell/mm^3; but when it was cultured it yielded a heavy growth of *Ps. pyocyanea*. On the eighth day the child collapsed and died. The autopsy showed that a *Ps. pyocyanea* pyaemia had been present, as well as focal cerebral encephalo-meningitis and suppurative otitis media. Cultures were made from the Casualty Department's stock bottles of Eusol, but these yielded neither *Ps. pyocyanea* nor any other pathogens, and the source of the infection was not disclosed.

A case of Ps. putida *infection.* An eight year old boy was admitted with a pyrexia of unknown origin, with some localized anginal lymphadenopathy, splenomegaly and hepatomegaly. None of the routine investigations provided specific help in the diagnosis except a blood culture, but unfortunately no growth was visible in this after 18 hr incubation. A second blood culture was then made, and when bone marrow was aspirated, to exclude a diagnosis of leukaemia, some of this marrow was also cultured. The child died 36 hr after the first blood culture was taken. Later examinations of the blood and marrow cultures showed that all had given pure growths of *Ps. putida*.

In the first blood culture there were 6 bacterial clumps/ml, but there were 300 in the second, as shown by the poured plate cultures. The slight scum on the fluid cultures of the blood and marrow, and the appearance of all the solid media cultures, made us think we would isolate *Ps. pyocyanea* but, as the cultures matured, neither the characteristic smell nor the pigment of *Ps. pyocyanea* was produced. There was no marked pigmentation of the colonies, but a very penetrating odour, like that of crushed daffodil stalks, came from all the cultures. At autopsy there was no purpura or other skin lesions such as are usually seen in a *Ps. pyocyanea* septicaemia untreated with Polymyxin B, but shallow ulcers were present in the upper intestinal tract and the spleen looked macroscopically as if it were from a rapidly fatal, but early case, of Hodgkin's Disease. The histology of the spleen failed to show any evidence of that disease, however. *Ps. putida* was grown from post mortem cultures of the spleen, an abdominal lymph gland and the lung. No virus agent was isolated from the post mortem specimens.

5. ROUTINE TECHNIQUES FOR THE ISOLATION OF *PS. PYOCYANEA*.

Any specimen likely to contain *Ps. pyocyanea* is planted on deoxycholate-citrate agar, as well as on all the routine media employed for the culture of throat, nose and pus swabs, as described by Rogers, Zinnemann & Foster (1960). If a specimen has not been inoculated on deoxycholate or MacConkey's agar plates, any colonies on prepared blood agar plates (Rogers & Heslop, 1948) or chocolate agar plates which resemble those of coli-aerogenes bacteria are subcultured for identification: this will often show that *Ps. pyocyanea* is present in small numbers. These routine media always include a tube of glucose infusion broth, and if *Ps. pyocyanea* is present this will usually show a surface scum after 18–24 hr incubation.

Table 2. *A representative result of the application of a routine technique for the isolation of* Ps. pyocyanea

Dilution in broth of original culture	Appearance of broth dilution*		Nos. of colonies from each of four drops on:		
	Turbidity†	Pellicle‡	Blood agar	MacConkey's agar	Deoxycholate-citrate agar
10^{-6}	++	+	9 14 17 17	8 12 13 16	4 8 11 14
10^{-7}	++	+	1 1 1 1	0 2 2 3	1 2 2 3
10^{-8}	++	–	0 0 0 1	0 0 0 1	0 0 0 0
10^{-9}	+§	–	0 0 0 0	0 0 0 0	0 0 0 0
10^{-10}	–	–	0 0 0 0	0 0 0 0	0 0 0 1

* After incubation for 18 hr at 37°. † Turbidity: ++, heavy; +, just visible; –, absent.
‡ Typical surface pellicle of *Ps. pyocyanea*: +, present; –, absent.
§ Both tube and colony were subcultured and proved to be *Ps. pyocyanea*.

To show that small numbers of *Ps. pyocyanea* are not suppressed on deoxycholate-citrate agar, viable counts of a freshly isolated culture were made on that medium, on 10% horse blood agar and on MacConkey's agar. The organism was subcultured into heart infusion broth and shaken at frequent intervals for 5 hr. Serial tenfold dilutions of this were made in warm infusion broth and immediately inoculated on to separate plates of the three media, four equal sized drops of each dilution being put on each plate with the same pipette held in a vertical position. The drops were not spread but allowed to soak into the media. All the plates, and the tubes containing the dilutions of the original culture, were incubated aerobically at 37° for 18 hr. A representative result is given in Table 2.

On deoxycholate-citrate agar medium very young cultures (12–18 hr old) of *Ps. pyocyanea* are not characteristic, but further incubation gives rise to typical, rather sticky, slightly pigmented colonies. After incubation for 18–24 hr on blood agar the colonies are also typical. In our experience *Ps. pyocyanea* is no easier to distinguish on Cetrimide agar plates (Lowbury & Collins, 1954) than on deoxycholate media. Moreover, when our routine specimens are planted on Cetrimide plates many strains of *E. coli* grow, whereas these are suppressed when the materials are inoculated on to deoxycholate-citrate agar. But it should be noted that we did not employ ultraviolet light (Lowbury, 1951) to help to distinguish between colonies of *E. coli* and *Ps. pyocyanea* on any of these media.

6. ACKNOWLEDGMENTS

I am grateful to Dr. W. C. Smallwood and Dr. W. H. Cant for permission to quote details of their cases. The identification of *Ps. putida* was made by Dr. B. Lacey, to whom I offer my sincere thanks.

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Notes added in proof

1. Section 3. Chlorhexidine, 1:1000, has now proved to be an effective antiseptic for use in the bottles attached to electric suction machines.

2. Section 5. Since the *Pseudomonas-Achromobacter* Symposium was held, Kovacs' oxidase test has been used and found valuable for the differentiation of *E. coli* and *Ps. pyocyanea*, as suggested by speakers in the Discussion.

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The Editors of The Journal of Applied Bacteriology *do not accept responsibility for the Reports of the Society's Proceedings. Abstracts of papers read are published without verification of the scientific content. As a rule where no abstract follows the title the paper read has formed the basis of a full length paper in the* Journal.

THE SOCIETY FOR APPLIED BACTERIOLOGY

WINTER MEETING

The Winter Meeting was held in the Barnes Hall of the Royal Society of Medicine, 1, Wimpole Street, London, W.1, on Wednesday, 13 January, 1960. In the morning there was a short business meeting, followed by a paper reading session. In the afternoon there was a Joint Meeting with the Microbiology Group of the Society of Chemical Industry, when the subject under discussion was 'Methods for the Evaluation of the Antibacterial Activity of Surface Active Compounds'.

Morning Session. The following papers were read:—

Further Studies on Disinfection of Cows' Teats Prior to Milking. By D. A. McKenzie and A. C. Hunter (*The North of Scotland College of Agriculture, Aberdeen*).

Little difference was found in the disinfecting efficiency of hypochlorite and quaternary ammonium disinfectants when used at 200 p/m for disinfecting cows' teats. Hypochlorite was slightly more effective in eliminating coli-aerogenes organisms. There was no evidence of greater survival of other Gram-negative species with the quaternary disinfectant. Micrococci constituted the main surviving species and, in summer, coagulase positive staphylococci formed a considerable proportion of these.

The Influence of the Growth Medium on the Demonstration of a Serological Group D Reaction by Faecal Streptococci. By T. F. Medrek* (*Low Temperature Research Station, Cambridge*).

Initial difficulties in serologically grouping the faecal streptococci isolated from the faeces and colon of cattle and sheep led to an investigation of the serological group D reaction using Lancefield HCl extracts.

A prerequisite for unequivocal serological group identification of certain group D streptococci is growth under special conditions. *Streptococcus bovis*, *Strep. durans*, and *Strep. faecium* require a medium in which growth of the organisms results in a low final pH, usually 4·0–4·3. It was found that the important factor is a glucose concentration of 0·5–1·0%, adjusted according to the buffering potentiality of the given medium. It is essential that these media support adequate growth, e.g. unbuffered Lemco broth (Shattock, 1949) or buffered Todd-Hewitt broth (see Swift, 1948).

It was theorized that the effectiveness of vaccines for preparing potent group D antisera may be similarly influenced by the composition of the medium.

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* The present address of the author is The Rockefeller Institute, New York 21, New York, U.S.A.

Techniques for Measuring Bacterial Penetrability of Paper and Similar Materials.

By J. G. DAVIS, G. ANNE HOWE and PAMELA STUBBS (*J. G. Davis & Partners, 9, Gerrard Street, London, W.1*).

Experiments were designed to measure the bacterial penetrability of paper with particular regard to toilet papers. *Salmonella*, a common organism causing food poisoning, is a faecal contaminant and is most likely to be transferred by the hands, which can easily become contaminated after defaecation. It was with this problem in mind that these investigations were made.

Eleven makes of paper were used, comprising most of the well known brands. These were of three main types: glazed, absorbent and crepe. The investigations were divided into five series and, following exploratory trials, the techniques adopted were as follows. (1) Drops of a bacterial suspension were allowed to fall from a height of 1 in. on to 1½ in. squares of paper previously cut and placed on a poured and hardened agar plate. The paper was removed after 5 sec and the plates incubated and colonies counted. (2) A pellet of soft artificial faeces impregnated with a 10% suspension of *E. coli* I was left in contact with 1½ in. squares of paper on an agar medium for 5 sec. (3) As for (2) but using firm artificial faeces. (4) As for (2) but using soft real faeces impregnated with *Salmonella paratyphi* B. (5) As for (4) but using firm real faeces.

Tests were made using single, double and treble thicknesses of paper. Yeastrel milk agar was used in experiments 1–3, and the bacterial suspensions were grown in nutrient broth. Wilson & Blair (modified) and MacConkey's agar (no. 3) were used for experiments 4 and 5.

Before commencing the tests it was established that all the papers were virtually sterile.

The conclusions reached from these series of tests were that one thickness of paper gave inadequate protection from bacterial penetration. In only a few cases did two thicknesses afford any protection, but three thicknesses proved to be a fairly effective barrier. Impregnation with germicides had no beneficial effect. An even textured and closely woven paper appeared to be the most important feature in preventing bacterial penetration.

Photomicrographs of the papers confirmed the conclusions of the bacteriological tests.

The Relationship Between the Mortality of a Bacterial Suspension and its Relative Viability on Different Media.

By N. D. Harris and J. P. Richards (*School of Pharmacy, Chelsea College of Science and Technology, London, S.W.3*).

Counts of phenol treated *Escherichia coli* on nutrient broth solidified with New Zealand agar and containing iron or chromium (0·00185 M) were higher than on the control agar without salts and the response was greater when the mortality of the suspension was higher. The regression of the logarithm of the response (the count on the treated medium divided by the count on the control) on the logarithm of the viable count on the control was linear. Similar results were obtained with *Pseudomonas aeruginosa* treated with phenylmercuric nitrate and counted on broth solidified with Japanese agar (control) and this medium containing iron, or prepared with New Zealand agar.

This relationship can be derived theoretically where the time-log survivor curves on two media are divergent. Thus it is important to have constant mortality levels if responses in different experiments of this type are to be compared quantitatively.

Some Aspects of the Microflora of Pasteurized Milk After Holding Under Refrigeration.

By A. Willsens and A. de Vleeschauwer (*Rijkslandbouwhogeschool, Leerstoel voor Technologie, Gent, Belgium*).

The bacterial quality of market milk is dependent on numerous different factors, e.g. the length and temperature of storage and also, but not least, on the initial flora.

The purpose of the present investigation was to obtain further information on the development of the initial flora of pasteurized milk held at usual refrigeration temperatures. Samples of bottled pasteurized milk were examined within 5 hr of leaving the dairy and after storage at 5° for 3, 6 and 10 days. A total of 463 representative cultures from the various groups of organisms isolated were subjected to more detailed study.

The following coli-aerogenes bacteria were encountered: *Citrobacter freundii*, *Aerobacter aerogenes* and *Paracolobactrum aerogenoides*. The organism occurring most commonly in samples both before and after refrigeration, however, appeared to belong to an anaerogenic group. The numbers of these increased on prolonged storage.

After refrigeration most samples were found to contain a few strains of *Aeromonas*, which have been classified by Dr. B. P. Eddy as *Aer. liquefaciens* and *Aer. formicans*.

The organisms which increased most on prolonged storage were *Pseudomonas* spp. Most of these proved to be *Ps. fluorescens* but occasionally *Ps. putrefaciens* strains were encountered. Other species were present but were difficult to classify. No easily identifiable species of *Achromobacter* were isolated but some organisms, which morphologically and physiologically closely resembled the so-called *Bacterium anitratum*, were included in this genus. Few *Flavobacterium* spp. were detected.

Refrigeration gave rise to an increase in the numbers of organisms which morphologically were identical to *Arthrobacter* as described in Bergey's *Manual*. The physiological characters were different, however, which made classification of these organisms difficult.

Of the micrococci isolated some corresponded to *M. candidus* and *M. luteus*; no coagulase positive strains were detected.

Streptococcus lactis strains were easily isolated on the TITG medium of Barnes (1956). Two types of enterococci were obtained after enrichment: *Strep. faecalis* and *Strep. faecium*. Strains of *Leuconostoc* closely resembling *Leuc. dextranicum* were isolated. The few cultures of *Microbacterium flavum* and *Micro. lacticum* which were isolated before refrigeration were not detected after refrigeration because they were outgrown by other organisms. This was also the case with *Kurthia zopfii*.

Only the spores of *Bacillus* spp. were determined. Practically the same flora was present before and after refrigeration. *B. pumilus* and *B. cereus* var. *albolactis* occurred in all samples (the latter only after enrichment); *B. cereus*, *B. cereus* var. *mycoides*, *B. subtilis* and *B. licheniformis* occurred in some of the samples only.

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Bacterial Multiplication in Gas-Packed Wiltshire Bacon. By M. INGRAM (*Low Temperature Research Station, Cambridge*). (See *J. appl. Bact.* **23**, 206).

Some Preliminary Observations on the Microbiology of Broiler Chickens.

By M. WOODBINE, A. SEAMAN and KATHLEEN N. SMITH (*Microbiological Unit, Department of Agricultural Sciences, University of Nottingham*).

Following a supplier's complaint, samples of birds were taken at the chicken processing factory and bacteriologically assessed by swabbing the skin and gut cavity, and streaking the suspension on to blood agar (incubated at 37°) and nutrient agar (incubated at 22 and 37°). The qualitative results indicated that: (1) the bird complained of was heavily contaminated, but frozen birds of the same consignment were satisfactory; (2) in processing, the skins of the birds in the first cooling tank were heavily contaminated, and the contamination increased or was maintained during overnight storage in iced water (a condition worsened by poor quality ice); but the bacterial load was decimated by the freezing process; (3) storage of the frozen carcases in the freezer compartments of domestic refrigerators was satisfactory for up to 14 days and storage on the cool sides of the same refrigerators was satisfactory for 1 to 2 days; (4) birds cooked and held under cool conditions were satisfactory for at least 2 days. The processed carcases, therefore, would deteriorate rapidly unless they were kept in a hard frozen condition. Some attention to processing hygiene would be worthwhile in the particular conditions examined.

Afternoon Session. The following papers were read at the Joint Meeting with the Microbiology Group of the Society of Chemical Industry on the subject of

Methods for the Evaluation of the Antibacterial Activity of Surface Active Compounds

Technical Aspects of the Problem. By J. G. DAVIS (*Dr. J. G. Davis & Partners, 9, Gerrard Street, London, W.*1). (See *J. appl. Bact.* **23**, 318).

The Mechanism of the Bactericidal Action of Synthetic Surface Active Compounds. By B. A. NEWTON (*Medical Research Council Unit for Microbiology, Biochemical Laboratory, University of Cambridge*). (See *J. appl. Bact.* **23**, 345).

The Assessment of the Activities of Surface Active Agents for Use in the Catering Industry. By BETTY C. HOBBS (*Central Public Health Laboratory, London, N.W.*9). (See *J. appl. Bact.* **23**, 350).

The Assessment of Surface Active Agents as Disinfectants in Milk Production and Processing. By CHRISTINA M. COUSINS (*National Institute for Research in Dairying, University of Reading*). (See *J. appl. Bact.* **23**, 359).

ANNUAL GENERAL MEETING AND CONFERENCE

The 30th Annual General Meeting and Conference was held in Neuadd Reichel, University College of North Wales, Bangor, on 5, 6 and 7 July, 1960. Members assembled on Monday, 4 July, and the following day was devoted to paper reading sessions in the morning and afternoon, followed by the Annual General Meeting. On Wednesday, 6 July, the morning was devoted to part of a Symposium on '*Pseudomonas* and *Achromobacter*', the afternoon to excursions to Snowdonia or Anglesey and the evening to the Annual Society Dinner. Thursday, 7 July, was occupied by the Symposium, which included an evening session devoted to a general discussion on differential techniques and methods of isolation.

The following papers were read on 5 July:—

The Microflora of Raw Milk as Determined by Plating on Yeastrel Milk Agar Incubated at 30°. By S. B. THOMAS, PHYLLIS M. HOBSON, ELIZABETH R. BIRD, KAY P. KING, R. G. DRUCE and D. R. COX (*National Agricultural Advisory Service, Trawscoed, Aberystwyth*).

The classification of the types of bacteria commonly found in 87 farm milk supplies, examined within 8–10 hr of production, was done by plating on Yeastrel milk agar incubated for 72 hr at 30°, and picking 24 colonies at random from each countable plate for detailed study.

Although the results indicate some general trends, such as the predominance of micrococci in low count milk (<20,000 colonies/ml), the codominance of streptococci, thermoduric corynebacteria and micrococci in milk with colony counts between 20,000 and 200,000/ml, and the dominance of *Achromobacter* and streptococci in the high count milks (200,000 to 5 million colonies/ml), it is evident that several milk samples, irrespective of colony count level, had a characteristic individual microflora. Thus, some of the low count samples contained an appreciable proportion of aerobic sporeformers, whereas coli-aerogenes organisms or thermoduric corynebacteria were dominant in some of the middle group samples. Even the high count group included some samples with an individualistic microflora dominated by either coli-aerogenes organisms, fluorescent pseudomonads or flavobacteria.

The 2,065 cultures were composed of micrococci, 38%; streptococci, 15%; Gram-positive rods (mainly thermoduric corynebacteria), 11%; coli-aerogenes organisms, 7%; other nonpigmented Gram-negative rods, 11%; pigmented Gram-negative rods, 10%; and aerobic sporeformers, 5%. The remaining 3% were streptomycetes and unclassified types.

Types of Psychrophilic Micro-Organisms in Refrigerated Raw Milk.

By S. B. Thomas and R. G. Druce (*National Agricultural Advisory Service, Trawscoed, Aberystwyth*).

Samples of raw farm milk supplies were held for 72 hr at 3–5° and plated on Yeastrel milk agar incubated for 10 days at 3–5°. Five colonies were picked at random from the countable plate for each of 51 samples and 235 of these cultures were studied in detail.

The psychrophilic microflora was dominated by Gram-negative organisms of the *Pseudomonas-Achromobacter* group. Thus, 58% of the 235 cultures were nonfluorescent pseudomonads or *Achromobacter* and 37% were fluorescent pseudomonads. The remaining 5% included five strains of *Arthrobacter*, five coli-aerogenes organisms, two *Alcaligenes*, one *Flavobacterium* and one yeast.

No micrococci were isolated at 3–5° from the refrigerated milk samples, and it is possible that in earlier references to the presence of psychrophilic micrococci they may have been confused with the coccoid stage of *Arthrobacter* which appears on agar within 24 hr at 22°.

Only 24 cultures formed colonies on Yeastrel milk agar in 48 hr at 37°. With the exception of 2 *Arthrobacter* and 2 *Achromobacter* or nonfluorescent pseudomonad cultures, all formed colonies in 72 hr at 30°.

Only 4% had an optimum growth temperature in glucose broth around 37°, whereas it was near to 30° for 61%; of the remainder, 8% grew best between 22° and 30° and 27% around 22°. None survived laboratory pasteurization in milk at 63·5° for 30 min.

Most of the cultures were strongly lipolytic and proteolytic: 90% hydrolyzed tributyrin, 80% liquefied gelatin, 51% were caseolytic on milk agar, and 52% produced a proteolytic fermentation in litmus milk at 22°.

These results indicate that the organisms which develop at 3–5° in raw milk may, in the absence of acidification, be capable of bringing about extensive breakdown of the fat and protein fractions and subsequent spoilage of the product.

Microbiological Aspects of Domestically Refrigerated Ice-Cream. By A. Seaman, Kathleen N. Smith and M. Woodbine (*Microbiological Unit, Department of Agricultural Sciences, University of Nottingham*).

A series of ice-creams from different manufacturers were examined bacteriologically before and after subjection to diverse conditions of domestic refrigeration. Samples were taken from both the surface and cross section of the ice-cream blocks, from blocks loosely or firmly closed, from blocks kept on the bottom or in the body of the freezer unit, and from blocks maintained at different temperatures. The results, broadly, were those which might have been anticipated in that ice-cream maintained at the 'coldest' setting and on the bottom of the freezer was good in all respects for 2 to 3 weeks. All the blocks were satisfactory, on a bacteriological basis, and there was little difference also in the surface or cross sectional samples, or in the closely or loosely packed samples.

Autoradiographic Techniques in Microbiology. By Erna Grossbard (*The Grassland Research Institute, Hurley*).

A method to study the uptake and incorporation of certain elements by micro-organisms utilizing radioactive tracers was discussed. The location of tracers was detected by autoradiography by a modification of Pelc's stripping film technique. The test organisms were *Bacillus megaterium*, *Lineola longa*, *Staphylococcus aureus*, *Streptomyces griseus* and an unidentified cellulolytic species of *Streptomyces*. These were cultured in glucose broth containing the nuclides (radioisotopes) ^{35}S, in the form of sulphates and ^{14}C, in glutamic acid or cellulose. A smear of the organisms was covered with a thin photographic emulsion on a gelatin base (the stripping film), exposed for 2 or 4 weeks and then processed. Since processing rendered the film transparent, microscopic examination was possible. The location of the tracer could be deduced from the presence of grains of reduced silver on the film as a result of the ionizing radiations emitted from the nuclides incorporated in the cells. These grains situated on the film are thus superimposed over labelled cells.

All the organisms seemed to incorporate the ^{14}C atom from the glutamic acid. The cellulolytic *Streptomyces*, cultured on a mineral medium containing labelled cellulose as its sole source of carbon, showed considerable accumulation of ^{14}C especially in the conidia. ^{35}S was supplied only to *L. longa*, *S. griseus* and *B. megaterium*. While the autoradiograms of the other two showed incorporation of the sulphur after 14 days exposure to the film, the results with *B. megaterium* were not clear cut. The evidence so far would suggest that, given a supply of organic sulphur as provided in the culture medium, this organism did not utilize inorganic sulphur. This point is being investigated more fully.

A method of dark field photomicrography of autoradiograms was demonstrated which rendered the silver grains very prominent though it reduced the definition of the underlying cells.

As compared with standard counting techniques by electronic instruments, autoradiography has the advantage of allowing microscopic observation of the uptake of nutrients and other substances by a small number of cells. A quantitative measure of uptake may be obtained by grain counting over a given area. This technique does not require expensive apparatus and is simple though rather slow.

The Viability on Different Agar Media of Bacteria Treated with Phenols.

By N. D. Harris and S. E. Jacobs (*Bacteriology Laboratories, Imperial College of Science and Technology, London, S.W.7*).

Suspensions of *Escherichia coli* and *Staphylococcus aureus*, either untreated or exposed to phenol, *o*-cresol or *p*-chloro-*m*-cresol, were counted on nutrient broth solidified with New Zealand agar (control), on the same medium which had been treated with Norit charcoal or salts (all at 0·00185 M), and on broth solidified with Japanese agar with or without similar treatments.

With untreated cells the counts were similar on all the media, but colonies of *Staph. aureus* were always larger when Japanese agar was used and often when the control medium had been treated. In contrast, the counts on the various media of cells of both species which had been treated with any of the bactericides differed markedly. The inclusion of Norit or the chlorides of iron, aluminium, chromium and (especially) magnesium, resulted in counts higher than on the control. The use of Japanese agar also resulted in high counts and the inclusion of ferric chloride in this medium led to the highest response of all.

The results are taken to indicate the presence in agar of substances inhibitory to untreated *Staph. aureus* cells and to damaged cells of both species.

Effect of Different Carbon Substrates and Cations on the Sporulation of Yeasts.

By R. R. Fowell (*Distillers Co. Ltd., Epsom*).

Metal salts of organic acids are useful sporulation media for yeasts as they act as buffers covering the pH range for optimal sporulation. Thus, when sodium acetate agar was inoculated with different yeasts, the pH shifted to the optimal value for sporulation (confirmed by use of buffered slants); this varied from one yeast to another over the range 6·8–8·0.

In general acetate proved more favourable to sporulation than pyruvate, succinate, citrate, lactate, glutamate and tartrate, particularly for 4-spored asci.

Of 13 yeasts tested on 0·01% (w/v) glucose agar, buffered at optimal pH values for sporulation, 6 sporulated better than on sodium acetate agar; but, with one exception, fewer 4-spored asci were produced on glucose agar.

The recent demonstration (McClary *et al.* 1959) that potassium is superior to sodium in stimulating sporulation was confirmed; but, for yeasts that sporulate well on sodium acetate agar, potassium was found less effective, the proportion of

4-spored asci being unaffected or even slightly depressed. Magnesium, with potassium, improved sporulation further but only in certain yeasts. As a general sporulation medium, 0·5% (w/v) sodium acetate, 0·23% (w/v) KCl and 1·5% (w/v) agar is recommended.

Buffering of sodium acetate agar usually improved sporulation due to potassium in the phosphate buffers used; when potassium chloride was added, however, buffering proved unnecessary. Addition of potassium to glucose agar had little effect on sporulation. Only 4 out of 13 yeasts sporulated better on buffered glucose agar than on acetate-KCl agar; and on average twice as many 4-spored asci were produced on this medium compared with glucose agar. Acetate media must therefore be regarded as superior to glucose agar because their efficiency, as sporulation media, unlike glucose agar, can be boosted by incorporation of potassium and, sometimes, magnesium.

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McCLARY, D. O., NULTY, W. L. & MILLER, G. R. (1959). Effect of potassium versus sodium in the sporulation of *Saccharomyces*. *J. Bact.* **78**, 362.

The Soluble Antigens of *Azotobacter*. By J. R. NORRIS and L. NAGY (*Department of Bacteriology, University of Glasgow*).

Antisera, prepared against typical strains of 6 *Azotobacter* species by injecting whole cell suspensions into rabbits, were used to study the nature and distribution (both within and between species) of surface antigens.

Agglutinogens showed strain or subspecies specificity in *Az. agilis*, *Az. macrocytogenes*, *Az. chroococcum* and *Az. beijerinckii*. Species specific agglutinogens were detected in *Az. vinelandii* and *Az. insigne*. An antiserum to *Az. agilis*, although agglutinating only the homologous strain of that species, gave a low titre agglutination with each of five strains of *Az. vinelandii*.

Extraction in saline at 37° for 30 min yielded antigenic extracts which were studied by precipitin and gel diffusion techniques. Such extracts usually contained more than one antigen. *Az. agilis*, *Az. vinelandii* and *Az. insigne* each had distinct, species specific, saline soluble antigens. *Az. macrocytogenes* had a strain specific soluble antigen. There was considerable cross reaction between extracts of *Az. chroococcum* and *Az. beijerinckii* strains and the corresponding antisera.

Suspending cells in their homologous antisera and observing them under the phase contrast microscope (cf. Tomcsik, 1956) revealed marked capsular reactions in *Az. agilis*, *Az. macrocytogenes*, *Az. chroococcum* and *Az. beijerinckii*, thus indicating that some at least of the soluble antigens were situated in the capsule of the living cell. Such capsular reactions proved to be largely strain specific.

It was concluded that *Az. agilis*, *Az. vinelandii*, *Az. insigne*, *Az. macrocytogenes* and *Az. chroococcum* should be considered as distinct species but that there appeared to be no clear cut antigenic distinction between *Az. chroococcum* and *Az. beijerinckii*.

REFERENCE

TOMCSIK, J. (1956). Antibodies as indicators for bacterial surface structure. *Annu. Rev. Microbiol.* **10**, 213.

The *Azotobacter* spp. of Flannan Island. By J. R. Norris and D. E. Baird (*Department of Bacteriology and Department of Zoology, University of Glasow*).

Fifty samples of surface soil (5 from each of 10 sampling areas) were taken from Eilean Mor, the largest island of the Flannan Group which is situated some 18 miles off the west coast of Lewis in the Outer Hebrides. Each sample was tested for the presence of *Azotobacter* by means of a fluid enrichment technique using mannitol. ethanol and sucrose as alternative carbon sources.

Thirteen isolates were made of which 12 were *Az. chroococcum* and came from one sampling area situated inside the wall of the lighthouse, the only habitation on the island. In 1947 this area had been cultivated, although no fertilizer had been applied. The yield of vegetables had been very poor and the attempt at cultivation had not been repeated. There was no evidence of cultivation elsewhere on the island.

The other isolate, from the southwest headland, proved to be a strain of *Az. macrocytogenes* (Jensen, 1955), an organism so far found only in a Danish soil. Our isolate resembled Jensen's closely, differing from it in the nature of its capsular antigen and in its failure to produce a fluorescent, water soluble pigment.

The soils were peaty in nature but had relatively high surface pH values, probably as a result of constant drenching with sea spray. The mean pH within the lighthouse wall was exceptionally high at 7·2 and the sample yielding *Az. macrocytogenes* had a pH of 6·9. Potash and phosphate estimations showed the soils to be rich in these materials, probably as a result of extensive deposition of bird excrement.

Reference

Jensen, H. L. (1955). *Azotobacter macrocytogenes* n. sp., a nitrogen-fixing bacterium resistant to acid reaction. *Acta agric. scand.* **2**, 280.

Carbohydrate and Organic Acid Metabolism in *Pseudomonas aeruginosa* with Reference to the Nature of the Diauxic Effect. By W. A. Hamilton and E. A. Dawes (*Department of Biochemistry, University of Glasgow*).

Although the tricarboxylic acid cycle is constitutive in *Pseudomonas aeruginosa*, it has been shown that entry of various exogenous intermediates of the cycle into cells grown on glucose as sole carbon source is mediated by inducible permeases (Campbell & Stokes, 1951).

Cells grown on an acid of the cycle as sole carbon source display a similar period of induction before oxidation of glucose commences, and in both cases studies with chloramphenicol have shown that net synthesis of protein occurs during this induction period. Presumably in this second case the permease, or the intracellular metabolizing enzymes for glucose, or both the permease and the enzymes, are inducible.

Organic acids also inhibit glucose metabolism. When cells were inoculated into an ammonium salt medium containing glucose and an acid as carbon sources, diauxic growth occurred, but contrary to previously reported cases with *Aerobacter aerogenes* the acid was the preferential growth substrate. This inhibition extends to non-proliferating cell suspensions. Citrate-grown cells, induced with glucose and therefore able to metabolize both glucose and citrate when presented singly, did not utilize glucose in the presence of citrate until the concentration of the latter had fallen to a low level.

Ps. aeruginosa possesses enzymes for the direct oxidation of glucose to gluconic and 2-oxogluconic acids (Stokes & Campbell, 1951), and since isotopic studies suggest the operation of the Entner-Doudoroff (1952) pathway and also the preferential elimination of C_1 of glucose (Wang, Stern & Gilmour, 1959), we have assayed some of the enzymes characteristic of these pathways in extracts of glucose- and acid-grown cells. We have found these enzymes of glucose metabolism to be inducible.

We are now investigating the possible existence of a glucose permease and a possible site of inhibition of glucose metabolism by organic acids in the light of current theories of feed back inhibition and enzyme repression.

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STOKES, F. N. & CAMPBELL, J. J. R. (1951). *Arch. Biochem.* **30**, 121.
WANG, C. H., STERN, I. J. & GILMOUR, C. M. (1959). *Arch. Biochem. Biophys.* **81**, 489.

The Utilization of Glucose by Aerobic Gram-Negative Bacteria.

By R. G. BOARD and A. J. HOLDING (*School of Agriculture, Edinburgh*).

A medium was devised in which an acid reaction was produced by aerobic Gram-negative bacteria which were capable of utilizing glucose. The medium had the following composition: $NH_4H_2PO_4$, 0·5 g; K_2HPO_4, 0·5 g; yeast extract (Difco), 0·5 g; agar (Davis, New Zealand), 5·0 g; distilled water, 1 l.; bromothymol blue; and a mineral supplement; pH 7·2. The medium was dispensed in 10 ml amounts in ⅝ in. test tubes and sterilized by holding momentarily at 22 lb/in^2. A filter-sterilized aqueous solution of glucose was added to the sterile medium to give a final concentration of 0·5% (w/v). Cultures were stabbed into the medium and incubated at 27°.

The bacteria which produced an acid reaction in the medium showed a rapid oxygen uptake in respirometer studies using glucose as a substrate. The nonacid-producing bacteria showed an oxygen uptake no greater than that due to endogenous respiration and were apparently unable to utilize glucose. Carbon dioxide did not appear to be a major factor responsible for the acid reaction since neither the holding of the sterile medium in an atmosphere of this gas nor the presence of a solution of KOH along with growing cultures produced a reaction different from that in tubes held under normal conditions in air.

The bacteria had been isolated from soil and rotting eggs, or were obtained from culture collections. With these organisms, the medium has proved useful in the identification of *Alcaligenes* strains and of pseudomonads which apparently are unable to utilize glucose. For this purpose the medium seemed more suitable than that described by Hugh & Leifson (1953) in which some glucose utilizing bacteria did not produce an acid reaction.

Malonate, methanol and methylamine hydrochloride were utilized as sole sources of carbon, but not ethylamine or ethanol in a washed agar, mineral salts medium.

There appears to be a close relationship between the cotton spoilage bacterium, similar bacteria from plant material and soil, and *Protaminobacter ruber* and *Ps. methanica*. However, *Ps. methanica* as defined by Dworkin & Foster (1956) is an obligate methane or methanol utilizer not capable of growth on complex media, whereas the bacterium described by Kaneda & Roxburgh (1959), similar in many characteristics to those described above, grew on methanol and complex media but not on methane.

It seems that there exist in nature pink, one-polar flagellate, Gram-negative rod shaped bacteria which although morphologically alike differ in degree of physiological specialization (cf. Harrington & Kallio, 1960). The validity of the present classification is therefore questioned and also the existence of the genus *Protaminobacter*, existing as it does solely on the basis of ability to utilize alkylamines as sole source of carbon and nitrogen (cf. Slepecky & Doetsch, 1954).

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HARRINGTON, A. A. & KALLIO, R. E. (1960). Oxidation of methanol and formaldehyde by *Pseudomonas methanica*. *Canad. J. Microbiol.* **6**, 1.

KANEDA, T. & ROXBURGH, J. M. (1959). A methanol-utilizing bacterium. 1. Description and nutritional requirements. *Canad. J. Microbiol.* **5**, 87.

SLEPECKY, R. A. & DOETSCH, R. N. (1954). Comparison of isolated aliphatic amine degrading organisms with known *Protaminobacter* species. *Bact. Proc.* 44.

The following papers were read on 6 July in the Symposium on

Pseudomonas and *Achromobacter*

Introductory Remarks. By M. INGRAM and J. M. SHEWAN (*Low Temperature Research Station, Cambridge* and *Torry Research Station, Aberdeen*). (See *J. appl. Bact.* **23**, 373).

General Considerations on the Classification and Identification of Gram-Negative Asporogenous Rods with Particular Reference to Pseudomonadacae. By J. M. SHEWAN, G. HOBBS and W. HODGKISS (*Torry Research Station, Aberdeen*). (See *J. appl. Bact.* **23**, 379).

This paper was followed by three relevant contributions:—

Taxonomy of the *Pseudomonas-Achromobacter* Group Using a Computer Method. By J. LISTON (*University of Washington, Seattle 5, U.S.A.*). (See *J. appl. Bact.* **23**, 391).

Computation of Similarities Between Strains of *Pseudomonas* and *Achromobacter* Isolated from Chicken Meat. By MARGARET J. THORNLEY (*Low Temperature Research Station, Cambridge*). (See *J. appl. Bact.* **23**, 395).

The Interpretation of Flagella Stains. By W. HODGKISS (*Torry Research Station, Aberdeen*). (See *J. appl. Bact.* **23**, 398).

Carbohydrate Metabolism and Localization of Enzymes in Micro-Organisms Related to *Pseudomonas*. By J. DE LEY (*Microbiology Laboratory, State University, Gent, Belgium*). (See *J. appl. Bact.* **23**, 400).

Differential Techniques and Methods of Isolation of *Pseudomonas*. By K. KLINGE (*Hygienisch-backteriologisches Institut, University of Erlangen*). (See *J. appl. Bact.* **23**, 442).

The following papers were read on 7 July:—

The *Pseudomonas-Achromobacter* Groups in Fresh and Spoiling Fish. By J. M. SHEWAN, G. HOBBS and W. HODGKISS (*Torry Research Station, Aberdeen*). (See *J. appl. Bact.* **23**, 463).

This paper was followed by one relevant contribution.

Bacterial Flora of Fish from the Pacific Ocean. By J. LISTON (*University of Washington, Seattle 5, Washington*). (See *J. appl. Bact.* **23**, 469).

Relationships of Organisms of the Genus *Pseudomonas* to Spoilage of Meat, Poultry and Eggs. By J. C. AYRES (*Food Processing Laboratory, Iowa State University*). (See *J. appl. Bact.* **23**, 471).

The Effect of Antibiotics and Irradiation on the *Pseudomonas-Achromobacter* Flora of Chilled Poultry. By M. INGRAM, MARGARET J. THORNLEY and ELLA M. BARNES (*Low Temperature Research Station, Cambridge*). (See *J. appl. Bact.* **23**, 487).

Mechanism of the Multiplication of *Pseudomonas* in the Egg. By J. BROOKS (*Low Temperature Research Station, Cambridge*). (See *J. appl. Bact.* **23**, 499).

Distribution and Ecology of Gram-Negative Organisms Other than Enterobacteriaceae in Fresh Water and Well Waters. By VERA G. COLLINS (*Freshwater Biological Association, Windermere*). (See *J. appl. Bact.* **23**, 510).

The Properties and Classification of the Predominant Gram-Negative Bacteria Occurring in Soil. By A. J. HOLDING (*School of Agriculture, Edinburgh*). (See *J. appl. Bact.* **23**, 515).

The Role of *Pseudomonas* in Plant Disease. By A. M. PATON (*Department of Bacteriology, University of Aberdeen*). (See *J. appl. Bact.* **23**, 526).

***Pseudomonas* Infections in a Children's Hospital.** By K. B. ROGERS (*The Children's Hospital, Birmingham*). (See *J. appl. Bact.* **23**, 533).

The following participated by invitation in an Evening Session: ELLA M. BARNES, J. M. SHEWAN, J. C. AYRES, K. KLINGE, VERA G. COLLINS, A. J. HOLDING, A. M. PATON, EVE BILLING, A. C. HAYWARD and W. HODGKISS.

AUTUMN MEETING, 1960

For the Autumn Meeting of the Society, held on Tuesday, 25 October, 1960, a visit was paid to Rothamsted Experimental Station, Harpenden, by kind permission of the Director.

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